

**Protein content and protease activity in senescing roots and leaves of wetland  
monocot species with contrasting root turnover strategies.**

**By**

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fulfillment of the requirements for  
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## Abstract

Perennial herbaceous monocots in Northern Ontario wetlands can be classified in two distinct types of root turnover strategies: those with overwintering roots, and those with complete root mortality at the end of the growing season. All species have autumn-senescing leaves. The present thesis is part of investigations to understand adaptive advantages of the two strategy types, focusing on nutrient remobilization from senescing roots. Existing data on nutrient remobilization from senescing roots is based on changes in element content in dying roots, and do not differentiate between remobilization and leaching out. Root protein content and aminopeptidase activity was assessed for garden-grown plants of six species from September to November, three species with autumn-senescing roots (*Rhynchospora alba*, *Sagittaria latifolia*, *Sparganium americanum*) and three with overwintering roots (*Carex oligosperma*, *Iris versicolor*, *Scirpus microcarpus*). We hypothesized that protein degradation and protease activity would be higher in autumn-senescing roots. The results confirm the existence of two root turnover strategies, species with annual roots showing a decline in root protein content, while species with perennial roots did not show such a decrease. Leaf protein content decreased in all species but *C. oligosperma*, known to senesce late. Total root aminopeptidase activity per fresh mass decreased in species with annual roots, but not in those with perennial roots. In contrast to expectation, specific aminopeptidase activity did not change over time and did not differ between the strategies. We conclude that nitrogen remobilization is an active process in senescing roots, and in autumn occurs only in annual roots. However, temporal characterization of root enzyme activities requires more detailed investigations.

**Keywords:** Root lifespan, Remobilization, Senescence, Wetland Plant, Functional Traits

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# Table of Contents

<b>Abstract .....</b>	<b>iii</b>
<b>Acknowledgments .....</b>	<b>iv</b>
<b>List of tables .....</b>	<b>vi</b>
<b>List of Figures .....</b>	<b>vii</b>
<b>List of Appendixes .....</b>	<b>viii</b>
<b>1. Introduction .....</b>	<b>1</b>
1.1 What is Senescence? .....	1
1.2 Why does senescence happen?.....	3
1.3 How is senescence associated with ecological conditions? .....	5
1.4 How do leaves know when to senesce?.....	6
1.5 Root Senescence .....	6
1.6 Annual and perennial roots in perennial plants .....	8
1.7 Goal of this study.....	9
<b>2. Materials and Methods.....</b>	<b>10</b>
2.1 Study species and growth conditions .....	10
2.2. Harvests.....	13
2.3. Root mortality .....	15
2.4. Chemicals Analyses.....	15
2.4.1. Protein Quantification .....	15
2.4.2. Protease assays .....	16
2.5. Dry matter content.....	18
2.6. Statistical Analyses.....	19
<b>3. Results .....</b>	<b>20</b>
3.1. Dry matter content.....	20
3.2. Root mortality .....	22
3.3. Protein content in roots and leaves.....	25
3.4. Leaf and root aminopeptidase activity .....	31
3.5. Leaf and root carboxipeptidase activity .....	31
<b>4 Discussion .....</b>	<b>39</b>
4.1. Review of nitrogen remobilization from leaves. ....	40
4.2 Review of nitrogen remobilization from roots .....	43
4.3 Comparison of findings of this thesis with literature .....	44
<b>5. Conclusions .....</b>	<b>48</b>
<b>References.....</b>	<b>49</b>

## List of tables

Table	Description	Page No
1	Dates of the first and last harvests for both roots and leaves for each species in 2014.	14
2	Dry matter content for leaves and roots of the studied species (mean $\pm$ standard error; g g <sup>-1</sup> )	21
3	Results of nested ANOVAs (mixed model) testing leaf and root dry matter content (DMC). Leaf and root DMC as the dependent variables, respectively, and root strategy type (annual roots, perennial roots) as a fixed independent factor, and species as a random independent factor nested within strategy. *** Means significant with P< 0.001	21
4	Results of mixed model nested ANOVAs testing for leaf and root protein content per fresh mass. Protein content as the dependent variable, root strategy type (Strategy; annual roots, perennial roots) as fixed independent factor, and day of harvest (Day) as continuous independent variable. The model also contained species nested within strategy type as a random independent factorial variable. Number of observations: 75 for roots, 52 for leaves. Significance levels: *** P< 0.001, ** P< 0.01, * P<0.05	26
5	Results of mixed model nested ANOVAs testing leaf and root aminopeptidase activity per fresh mass. Aminopeptidase activity as the dependent variable, root strategy type (annual roots, perennial roots) as a fixed dependent factor, and species nested within strategy as a random independent variable. Number of observations: 65 for roots, 49 for leaves. *** Means significant with P< 0.001	32
6	Results of nested ANOVAs (mixed model) testing leaf and root amino peptidase activity per protein content. Amino peptidase activity as the dependent factor, root strategy type (annual roots, perennial roots) as fixed independent factor, and species nested within strategy type as a random independent factor, and day of harvest as continuous independent variable. Number of observations: 65 for roots, 49 for leaves.	32

## List of Figures

Figure	Description	Page Number
1	a picture of the pot temperatures (Red line) and air temperatures (black line) in the experimental garden from August 15 2014 to November 12 2014.	12
2	Percentage alive root length in the roots of the studied species in the autumn. Root vitality was determined by staining with, 2, 3, 5-Triphenyl Tetrazolium Chloride (TTC). A-C: species with perennial roots, D&E: species with annual roots.	23,24,25
3	Leaf and root protein contents of the six studied species plotted per fresh mass in course of autumn senescence. Regression lines indicated in blue (leaves) and red (roots). Solid lines $p < 0.15$ , dotted lines $p > 0.15$ . a-c: species with perennial roots, d-f: species with annual roots.	27-30
4	Leaf and root aminopeptidase activity for <i>Iris versicolor</i> in course of autumn senescence. a) Aminopeptidase activity per protein content. b) Aminopeptidase activity per fresh mass. Regression lines indicated in blue (leaves) and red (roots). Solid lines $p < 0.15$ , dotted lines $p > 0.15$ .	33
5	Leaf and root aminopeptidase activity for <i>Scirpus microcarpus</i> plotted per fresh mass in course of autumn senescence. a) Aminopeptidase activity per protein content. b) Aminopeptidase activity per fresh mass. Regression lines indicated in blue (leaves) and red (roots). Solid lines $p < 0.15$ , dotted lines $p > 0.15$ .	34
6	Leaf and root aminopeptidase activity for <i>Carex oligosperma</i> plotted per fresh mass in course of autumn senescence. a) Aminopeptidase activity per protein content. b) Aminopeptidase activity per fresh mass. Regression lines indicated in blue (leaves) and red (roots). Solid lines $p < 0.15$ , dotted lines $p > 0.15$ .	35
7	Leaf and root aminopeptidase activity for <i>Rhynchospora alba</i> plotted per fresh mass in course of autumn senescence. a) Aminopeptidase activity per protein content. b) Aminopeptidase activity per fresh mass. Regression lines indicated in blue (leaves) and red (roots). Solid lines $p < 0.15$ , dotted lines $p > 0.15$ .	36
8	Leaf and root aminopeptidase activity for <i>Sparganium americanum</i> plotted per fresh mass in course of autumn senescence. a) Aminopeptidase activity per protein content. b) Aminopeptidase activity per fresh mass. Regression lines indicated in blue (leaves) and red (roots). Solid lines $p < 0.15$ , dotted lines $p > 0.15$ .	37
9	Root aminopeptidase activity for <i>Sagittaria latifolia</i> plotted per fresh mass in course of autumn senescence. a) Aminopeptidase activity per protein content. b) Aminopeptidase activity per fresh mass. Regression lines indicated in red (roots). Solid lines $p < 0.15$ , dotted lines $p > 0.15$ .	38

## **List of Appendixes**

<b>Appendix</b>	<b>Description</b>	<b>Page No</b>
<b>1</b>	Pictures for the garden at the beginning of the experiment in May (a), at beginning of harvesting in September (b) and at setting up the experiment in May (c).	59
<b>2</b>	Pictures for roots and leaves of all species at early and late harvests.	60,61
<b>3</b>	Pictures of roots after staining with TTC test in November at the last harvest to investigate root mortality.	62



# 1. Introduction

## 1.1 What is Senescence?

Senescence is a stage in plant growth at which the plant prepares for either death or partial death before dormancy over an unfavorable season (Thomas et al. 2009), such as the winter season in Northern Ontario. At this stage the plants undergo a process of remobilization of nutrients, mainly nitrogen and phosphorus (Côté *et al.*, 2002; Avila-Ospina et al. 2014), from the green leaves, and transportation of these nutrients to other parts of the plant that will stay alive but dormant. Such overwintering parts can be buds and other meristematic tissues, as well as stems and branches in deciduous trees, rhizomes and other below-ground storage organs in herbaceous perennial plants, or the seeds in case of annual plants (Maillard *et al.*, 2015). Coarse roots of trees overwinter (Regier *et al.*, 2010), but there are only a few studies on fine root survival over the winter, especially in perennial herbaceous species. For some species root survival over winter has been documented, for some a complete death (Shaver & Billings 1975, Ryser & Kamminga 2009), but physiological processes of senescence have so far not been documented. Fisher et al. (2002) did not find any programmed senescence in dying bean roots.

Leaf senescence in herbaceous perennials and deciduous trees happens in the fall season, at which the leaves lose the pigmentation, dehydrate, and eventually detach from the stem. The process of senescence at the cellular level involves subcellular and biochemical changes. At the subcellular level, organelles like chloroplasts differentiate into gerontoplasts (Matile et al. 1999; Keskitalo *et al.*, 2005). At the biochemical level

chlorophyll, carotenoids and xanthophylls degrade at different rates, with the former degrading faster than the latter two (Hörtensteiner 2006).

The degradation of pigments together with the structural and enzymatic proteins in the senescing leaves represents a nutritional economical value for the plants. When temperatures fall below the freezing point the fresh green leaves if left the way they are will freeze and die (Estrella and Menzel 2006; Vitasse *et al.*, 2009). By remobilizing the leaf macronutrients and then blocking the connection between the stem and leaves vessels at the abscission layer the plant is reducing nutrient losses due to the frost damage done to the parts that has high enough water content.

Nitrogen and phosphorus are among the nutrients that the plant can only acquire through absorbing from the soil via the roots. In most ecosystems, these nutrients are growth limiting (Aerts and Chapin 2000). Losing these two in the falling leaves would be a huge waste given that the plant invested a whole growth season collecting them.

By undergoing senescence the plants can recycle nutrients by remobilizing those in the leaves and resorbing them in overwintering organs. For deciduous trees in eastern Canada up to 71% of leaf nitrogen and 78% of phosphorus has been found to be remobilized in hardwood trees (Côté *et al.*, 2002).

During the process of degradation of the cellular components of the leaves proteins undergo proteolysis, a process at which peptides are eventually broken down into the composing individual amino acids before they can be carried and translocated from the leaves to other parts of the plant (Roberts *et al.*, 2012). The process is facilitated by enzymes whose transcription is triggered by environmental factors that regulate the genes

coding for those enzymes to be activated at the time of senescence (Buchanan-Wollaston, 1997; Bhalerao *et al.*, 2003; Buchanan-Wollaston *et al.*, 2003; Andersson *et al.*, 2004; Guo *et al.*, 2004). The regulation of the process of the senescence at the gene level will be discussed in section 3 of this introduction. The amino acids resulting from the process of proteolysis are used as building blocks of other enzymes, and structural and storage proteins in other places of the plant that are going to survive the fall, either as dormant plant parts as in the trees or as seeds in annual plants (Liu *et al.*, 2008).

## **1.2 Why does senescence happen?**

In annual plants senescence is the end of the vegetative sporophytic stage and a preparation of entering the dormant gametophytic stage (seeds) and is a clear manifestation of the alternation of generations. The gametophyte is composed of the embryo that has the potential to germinate and develop into a full gametophyte in favourable conditions, and the stored nutrients needed during the germination stage until the new seedling is ready for assimilating it's own food through photosynthesis.

In deciduous trees and herbaceous perennials the process of senescence takes place in order to preserve the nitrogen and phosphorus of the leaves and store them where the plant will use them the next growth season when the conditions are favourable. The senescence in annual plants aims at storing the nutrients, collected from the senescing parts, in the seeds with the minimum water content. This process is also known as seed filling. (Roberts *et al.*, 2012, Avila-Ospina *et al.*, 2014).

Other stresses can also cause plants to undergo senescence, such as drought (Munné-Bosch *et al.*, 2001) and insufficient light intensity (Brouwer *et al.*, 2012).

Drought, or water stress, can cause senescence to take place, with different processes than in naturally occurring senescence, distinguished by activation of different peptidases and by the presence of two extra polypeptides (60 and 69 kDa) (Khanna-Chopra *et al.*, 1999).

Shade is one of the factors that is known to induce senescence. In crowded canopies where the lower leaves receive little light, this shade triggers senescence in those leaves as a competitive adaptation strategy. In shade-induced senescence the plants remobilize the nitrogen, phosphorus, and other macronutrients trapped in the leaves in the shade and reuse them in new leaves at the top of the canopy where light is available (Saur *et al.*, 2000; Boonman *et al.*, 2006).

Plants also may show programmed cell death in response to pathogens, but in contrast to senescence, which aims to remobilize nutrients from the senescing organ, the goal of a programmed cell death is rapidly killing the cell to prevent the spread of the pathogen (Buchanan-Wollaston *et al.*, 2003). Nevertheless, infection of *Arabidopsis* mutants *vitamin c-1* with *Pseudomonas syringae* and *Peronospora parasitica* was associated with elevated levels of senescence-associated gene (SAG) transcripts, indicating a relationship between the pathogenic infection and the induction of senescence (Barth *et al.*, 2004).

Most spectacular process of senescence can be observed when leaves of deciduous trees senesce before the onset of winter in temperate climate zones, a phenomenon associated with a multitude of bright colours (Matile 2000). This transformation from green to yellow and/or red is due to the difference in the rate of degradation of the pigments responsible for these colours, with chlorophyll degrading faster than the

carotenoids (yellow), although in some cases beta-carotene was reported to be degrading parallel to chlorophyll while neoxanthin and xanthophyll took longer (Keskitalo *et al.*, 2005). The red colours are caused by *de novo* synthesis of anthocyanins as photoprotection for the senescing leaves (Guy & Krakowski 2003).

### **1.3 How is senescence associated with ecological conditions?**

Deciduous forests occur in climates where winter is too cold for leaves to survive without large investment in frost tolerance, and where the summer is long enough for photosynthesis to compensate for the annual loss of leaves. In such climates the length of day and the temperature are the main ecological factors that trigger the sequence of senescence (Fracheboud *et al.* 2009). The fluctuating temperatures at the end of summer and the beginning of fall makes it less influential in triggering senescence, with the length of photoperiod is believed to be a better determinant cue for the senescence to start (Way, 2011). Temperature will modulate the progress of senescence after it has started (Keskitalo *et al.*, 2005)

If summer is too short for production of new leaves and a payback of the investment, evergreen conifers dominate, such as in the boreal forest (Kikuzawa & Lechowics 2011). If the winters are mild and there is not need to discard the leaves, broadleaf evergreen forest dominate, such as in many subtropical zones where plant density and availability of light and nutrients are more involved on triggering local or organ specific senescence (Saur *et al.*, 2000; Boonman *et al.*, 2006).

Global warming effect on the length of the growth season has been studied. The increase in the growth season due to raising temperatures is thought to have little effect on the ability of the plant to synthesise more biomass due to the photoperiod signal that

triggers senescence regardless of the warmth in photoperiod-controlled tree species (Rohde *et al.*, 2011). However, raising temperatures have more effect on the time between growth cessation and the activation of buds to burst the next growth season (Rohde *et al.*, 2011).

#### **1.4 How do leaves know when to senesce?**

In perennial species, seasonal senescence responds to two main environmental signals: day length and temperature, with the former being the more reliable factor to trigger senescence because it's the most constant over the years. It is not well known how this works and further studies are needed. (Keskitalo *et al.*, 2005, Vitasse *et al.*, 2009). As a response to these two factors, among others, the cells in senescing tissues in the leaves start up regulating genes encoding known and potential proteases in order to start the proteolysis of leaves proteins starting with the chloroplasts and ending with the mitochondria and the nucleus (Roberts *et al.*, 2012). Beginning of senescence may show interspecific variation even under similar climate.

Global warming may delay onset of senescence in deciduous tree species sensitive to temperature, such as beech (Vitasse *et al.*, 2009). The increasing temperatures are causing longer canopy duration, which permits longer exposure to solar radiation and more photosynthesis. However, terrestrial C sink is thought to be negatively affected by the extended canopy duration due to increased respiration (Piao *et al.*, 2008; Way 2011).

#### **1.5 Root Senescence**

It is well known that leaves of all herbaceous species and deciduous trees senesce before the onset of winter. Little is known about the roots though (Iversen *et al.*, 2015),

and more information of root phenology is needed (Radville *et al.*, 2016). And this is especially true in temperate and colder regions because the decomposition of dead roots in such cold soils is going to be slow, given that the temperatures from the senescence point and until the next growth season are below zero, where microorganisms' decomposing activity will be minimal (Weintraub & Schimel, 2005).

It has been often assumed that most fine roots of herbaceous species die back for the winter (Eissenstat & Volder 2005). However Shaver and Billings (1975) found for several arctic graminoid species that their roots can live for several years. Ryser and Kamminga (2009) found for northern Ontario that roots of many herbaceous wetland monocotyledons perennials survive over the winter, while in one of the studied species lateral roots died in fall, and in another species both axile and lateral roots died at the beginning of winter. A screening of a number of species has shown that northern Ontario wetland monocots can be divided in species with annual roots, senescing each autumn, and perennial roots which overwinter (Gagnon 2014; Susara J.E. Marcotte, unpublished data). These species also show differences with respect to antioxidant enzyme activities in their roots (Yücel *et al.*, 2014).

It has not been clearly established whether there are active processes of senescence, i.e. nutrient resorption, from senescing roots. Many studies suggest the hypothesis of the lack of programmed death in roots during shoot senescence in herbaceous species. Nambiar (1987) reported for tree species such as *Pinus radiata* that root death in winter was not evidently linked to any programmed death based on the observation of little translocation of nutrients from senescent roots, and Fisher et al. (2002) did not find any indication of programmed senescence of bean roots when the shoot senesced. In contrast,

Simpson *et al.* (1983) reported resorption of nitrogen from roots into grains during the whole-plant senescence of the annual plant *Triticum* (wheat), comprising 16% of the total nitrogen resorption. Similar root nitrogen resorptions were also documented in perennial grasses and perennial trees by Woodmansee *et al.* (1981) and Meier *et al.* (1985), respectively. Resorption of macronutrients from the fine roots during senescence was reported in 40 subarctic species from aquatic, riparian, and terrestrial environments, although at lower rates than resorption from stems and leaves (Freschet *et al.*, 2009). However, they only measured the elemental content before and after senescence, and it is possible that the nutrients were only leaching out from decomposing roots. No one has shown yet an active senescence process in dying roots. Whether roots undergo senescence or not is complicated by different factors, such as the absence of abscission zones and the less obvious seasonality of root senescence.

### **1.6 Annual and perennial roots in perennial plants**

Perennial plants have vegetative structure which live for several years (Evert & Eichhorn 2013). Leaves of herbaceous perennials senesce at the end of the growing season (Watson & Li 2004), but for such species, roots may or may not survive the winter (Ryser & Kamminga 2009). The two strategies of wetland plants in northern Ontario with respect to their root turnover resemble the two strategies of trees, with deciduous and evergreen leaves. For leaves the ecological advantages of deciduous and evergreen strategies are well known (Kikuzawa & Lechowicz 2011), for roots not. As resorption of nutrients from senescing leaves is an important aspect of above-ground senescence, one may assume that resorption from senescing roots is an important aspect of a plants nutrient balance. Often more than half of plant biomass in roots (Jackson *et al.* 1996) .



### **1.7 Goal of this study**

The goal of this study is to find out whether or not there is an active nutrient resorption processes in senescing roots. The focus will be on finding clues about the process of nitrogen resorption by investigating protein content and peptidase activity in the roots late in the growing season. The idea is that if there is an increased peptidase activity in the autumn correlated with decrease in protein content that would indicate an active remobilization process for nitrogen. If positive, the investigations in this thesis would support the hypothesis that roots undergo programmed senescence similar in nature to the one observed in leaves. The taken approach is to compare species with known root senescence in autumn with species with roots which are not senescing as they survive the winter. The investigation will be conducted as a common garden experiment to minimize variation caused by local environmental factors.

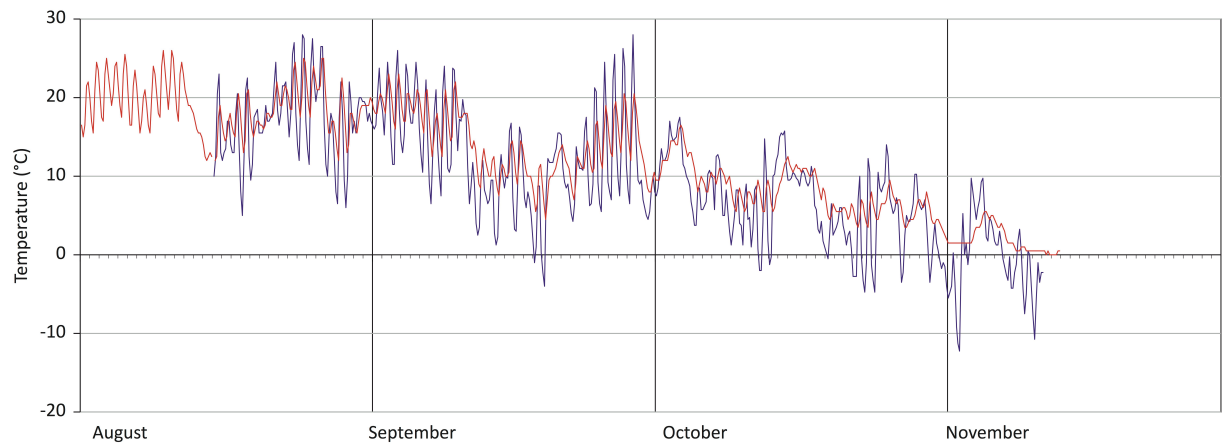
## 2. Materials and Methods

### 2.1 Study species and growth conditions

In order to understand the effect of root mortality on plant nutrient balance from senescing roots in the autumn, changes in their protein content and peptidase activity were investigated in course of the last months of the growing season. Six monocotyledonous plant species were investigated for this project, all-common in wetlands in the Sudbury region. Three species with fall-senescing root systems (*Rhynchospora alba* (L.) Vahl, *Sagittaria latifolia* Willd, *Sparganium americanum* Nutt.) and three species with overwintering root systems (*Iris versicolor* L., *Carex oligosperma* Michx., *Scripus microcarpus* J.Presl & C.Presl) were chosen for the study. These plants were started from vegetatively multiplied tillers, from plants collected in wetlands of the region in previous years. On May 6, 2014 20 replicate plants of each species were planted, 120 pots for all species in total. Each species had 20 replicate samples growing in 10-liter, 25 cm diameter pots, placed in pools filled to 20 cm depth with ground water in an experimental garden (Appendix 1). The substrate in the pots was *Sphagnum* peat moss with 0.5% (volumetric) composted sheep manure. The 120 pots were arranged equally in 9 pools of 0.75 m \* 1.60 m. During the growing season the pots were periodically weeded.

The temperatures were measured in the experimental garden at about 1 m height using iButton® temperature data loggers (DS1921G-F5# Maxim Integrated, San Jose, USA) protected from direct solar irradiance. The data loggers with a resolution of 0.5 °C were placed in small waterproof CPVC containers, recording the temperature every 255 minutes. Average temperatures between the planting (6 May) and last harvest (12

November) were 12.9°C for May, 17.4°C for June, 18.1°C for July, 17.2°C for August, 12.4°C for September, 6.3°C for October, and -0.8°C for November. The first night frost occurred on 18 September and the first day entirely below zero was 31 October (Fig. 1). In September there were 2 days with frost, in October 9 days and in November until the 12<sup>th</sup> there were 9 days with frost. Soil temperatures cooled more slowly, being around 10°C in early October and cooling down to 0°C by 11 November (Figure 1).



**Figure 1.** Temperatures of the experimental plants during the late growing season from August - November 2014. Blue line: Air temperature. Red line: Pot temperature.

## **2.2. Harvests**

After about three months of growth (May to mid August), the plants were harvested beginning on 14 August 2014 the second harvest being on 26 August. Unfortunately, these samples were mostly lost. After September 15, there were mostly two harvests a week until November 12. At last few harvests, the leaves of some species had fully senesced and were not harvested (Table1). At each harvest one replicate pot of each species was harvested. First, the whole plant was collected after thoroughly rinsing all substrate from its roots (Appendix 2). After that the roots were cut in pieces of about 3 cm and 5 samples of about 500 mg were collected. Also the leaves were cut in pieces of about 3 cm and 3 samples of about 500mg were collected for each plant. The samples were wrapped in aluminum foil, labeled and flash frozen in liquid nitrogen. Then the samples were stored them in a freezer at about -20 to -28 °C.

**Table 1.** Dates of the first and last harvests for both roots and leaves for each species in 2014.

Species	Roots		Leaves	
	1 <sup>st</sup> harvest	Last harvest	1 <sup>st</sup> harvest	Last harvest
<i>Iris versicolor</i>	SEP.15	NOV.12	SEP.15	NOV.4
<i>Scirpus microcarpus</i>	SEP.25	NOV.12	SEP.25	OCT.28
<i>Carex oligosperma</i>	SEP.16	NOV.12	SEP.16	NOV.12
<i>Sparganium americanum</i>	SEP.16	NOV.12	SEP.16	NOV.12
<i>Sagittaria latifolia</i>	SEP.19	OCT.10	SEP.19	SEP.19
<i>Rhynchospora alba</i>	SEP.15	OCT.30	SEP.15	OCT.30

### **2.3. Root mortality**

In this experiment, triphenyl tetrazolium chloride (TTC) was used as a staining solution method to assess root viability (Appendix 3). The surviving roots react with TTC by reducing the compound to red triphenyl formazan (TF), as seen in the methods published by Comas (*et al.* 2000). Roots were cut to pieces of about 2 cm length and incubated in 0.3% TTC with 10 mM glucose for approximately 2 hours at 30 °C and no light exposure (Ryser & Kamminga, 2009). The roots were taken out of the vials and stored in 50% ethanol. After that I measured the percentage of root length with red colour (living roots with TF) under the microscope (Figure 2 a, b, c, d, e).

### **2.4. Chemicals Analyses**

#### **2.4.1. Protein Quantification**

For protein extraction the method described in Guiboileau *et al.* (2013) was used after slight modifications. Samples were prepared for protein quantification in three steps. First, 500-1000 mg of the frozen samples were weighed and thoroughly ground while kept frozen with liquid nitrogen. Before grinding, PPVP (Polyvinylpyrrolidone) was added to the sample. Washed sea sand (Fisher Scientific, Fair Lawn, New Jersey, USA) was used as an abrasive at grinding. Second, the ground sample was transferred to a 5 ml centrifuge tube and 3000 µl of protein extraction buffer (25 mM Tris HCl pH 7.5) was added, while kept cold on ice container during my work. As the last step of protein extraction samples were centrifuged (TOMY-MRX-152 high speed refrigerated micro centrifuge) for 10 minutes at 15000-20000× g at a temperature of 4°C, and after which the supernatant was transferred to new tubes while keeping them on ice. The samples

were desalted using Sephadex G-25 in 25 mM Tris HCl extraction buffer pH 7.5, and centrifuging them twice, once for 3 minutes at 30× g and then followed for another 3 minutes at 300× g. One ml protein extract was incubated on ice bath for about 10 minutes, and spun for 3 minutes at 30× g to be followed for 3 minutes at 300× g at 4°C. The fluid was transferred to new tubes to be used for the protease assays.

To quantify the content of protein in roots and leaves, a protein standard was made by diluting 1 mg/ml BSA in tubes for concentrations of 0.1, 0.3, 0.5, 0.7, 0.9 mg/ml. Desalted extract was added on micro plates, 20 µl in each well, after which 250 µl of Bradford reagent was added in each well. The plates were incubated at room temperature for about 10 minutes after which the absorbance at 595 nm was read using Fluostar Optima (BMG Labtech GmbH, Ortenberg, Germany) micro plate reader.

#### **2.4.2. Protease assays**

##### **2.4.2.1. Aminopeptidase**

As substrate, 3.3 mM L-Leu-p-nitroanilide in 200mM Tris HCl pH 7.5, 1.7% DMSO was used. Prior to that a 2 mM solution in 100 mM Tris HCl and 1% DMSO was tried, but the measured enzyme activities were too low for proper detection. For the assay, 120 µl desalted extract was added in the wells with 120 µl substrate solution. Controls without substrate were run for each sample. Absorbance at 405 nm was measured for an hour every 2 minutes at 25 °C using Fluostar Optima (BMG Labtech GmbH, Ortenberg, Germany). Aminopeptidase activity was calculated as the slope of these absorbances over time, after deduction of the values in the control samples.



#### **2.4.2.2 Carboxipeptidase Assay**

##### **Substrate**

For the analysis of carboxypeptidases, the method of Guiboileau *et al.*, 2013 was slightly modified. The calibration solution consisted of 1mM glycine, as reagent 150 ppm TNBS (trinitrobenzene sulfonic acid) in 50mM Na borate pH 9.0. The original substrate, 2mM N-CBZ-phenylalanine in 100mM NaOAc pH 5.0, 2% DMSO did not work out, so I tried 1 mM and 10 mM N-CBZ-phenylalanine in 500mM NaOAc and 2% DMSO with pH 5.0. And a blank solution of 100 mM NaOAc buffer pH 5.0, 2% DMSO. The calibration solution was the same of value of 1mM glycine and reagent of 150 ppm TNBS (trinitrobenzene sulfonic acid) in 50mM Na borate pH 9.0. However, due to low enzyme activities using these concentrations, we modified the procedure to maximize the amount of obtained enzymes and used as substrate 26 mM N-CBZ-phenylalanine in 1.75mM NaOAc and 26% DMSO, with a pH 5.0.

##### **Method**

Glycine standard with two calibration curves with 0-50 nmol glycine (50,40,30,20,10 and 0 nmol glycine) were prepared, filling the wells with double distilled water up to 50 µl. The control consisted of 50µl of the darkest sample without substrate. The samples were analyzed with 40µl of sample and 10µl of substrate solution in each well. The microplate was covered and incubated for about an hour at 37 °C at room temperature. After that 150 µl of reagent was added and the plate was incubated for another hour at room temperature but in a dark place without light. After that the absorbance at 405nm was read using Fluostar Optima (BMG Labtech GmbH, Ortenberg, Germany).

## **2.5. Dry matter content**

To characterize the plants ecological behavior, leaf and root dry matter content (DMC) was assessed. Fresh mass of leaf and root samples was determined after drying their surface with paper towel, leaves after hydrating them to full turgor using the protocol of Ryser et al. (2008). Leaf and root dry masses were determined after drying them at 75°C for at least 24 hours.

## **2.6. Statistical Analyses**

Statistical analyses were conducted using R statistical package (R core team, 2014). Most variables were log transformed to attain normality. In order to compare the value of dry matter content of roots and leaves, ANOVAs were conducted for leaf and root DMC as the dependent variable, root type (annual roots, perennial roots), and species as a random independent variable. Linear mixed effects model were conducted to analyse the effects of the strategy types and time to leaf and root protein content and aminopeptidase activity, with the roots types (annual roots, perennial roots) as fixed dependent factor, species (6 species) as a random factor nested within the strategy types, and day of harvest as continuous variable. Additionally, the interaction factor day  $\times$  strategy type was included in the models.

### 3. Results

#### 3.1. Dry matter content

Both root dry matter content (DMC) and leaf DMC showed significant variation among the studied species (Tables 2, 3). In an overall comparison between species with either annual and with perennial root systems, it can be seen that species with perennial roots have a higher root DMC value than species with annual roots (Tables 2). The values for root DMC varied between  $0.06 \text{ g g}^{-1}$  for *S. latifolia* and  $0.24 \text{ g g}^{-1}$  for *S. macrocarpus*.

For leaf DMC, no such difference between the strategy types of annual vs. perennial roots could be concluded (Table 2). The highest DMC value and the lowest DMC value were found in species with annual root systems, *Sparganium americanum* and *Rhynchospora* respectively, and the DMC values for species with perennial species fall in the mid range. *S. latifolia* leaf DMC was not measured because the leaves of this species senesced before the harvests, and for *R. alba* only one plant could be measured. The values for leaf DMC were generally higher than those for root DMC, ranging between  $0.18 \text{ g g}^{-1}$  and  $0.35 \text{ g g}^{-1}$ .

**Table 2.** Dry matter content for leaves and roots of the studied species (mean  $\pm$  standard error; g g<sup>-1</sup>)

Species	Roots	Leaves
<i>Iris versicolor</i>	0.14 $\pm$ 0.007	0.20 $\pm$ 0.02
<i>Scirpus microcarpus</i>	0.24 $\pm$ 0.02	0.29 $\pm$ 0.01
<i>Carex oligosperma</i>	0.18 $\pm$ 0.03	0.33 $\pm$ 0.03
<i>Sparganium americanum</i>	0.11 $\pm$ 0.02	0.18 $\pm$ 0.01
<i>Rhynchospora alba</i>	0.09 $\pm$ 0.02	0.35
<i>Sagittaria latifolia</i>	0.06 $\pm$ 0.008	-

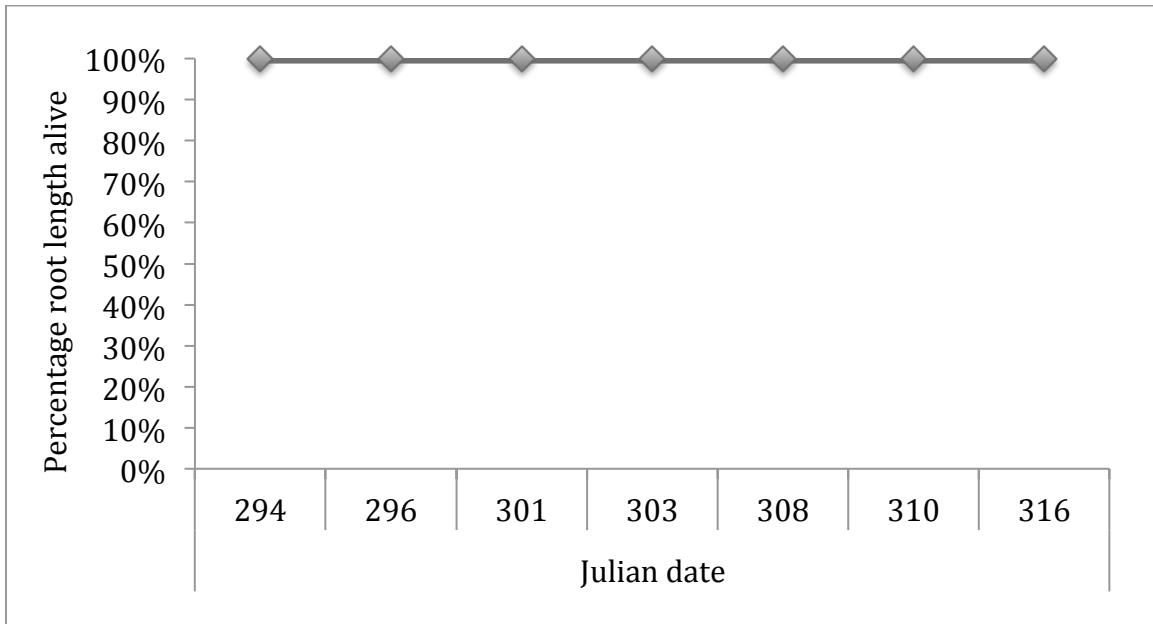
**Table 3.** Results of nested ANOVAs (mixed model) testing leaf and root dry matter content (DMC). Leaf and root DMC as the dependent variables, respectively, and root strategy type (annual roots, perennial roots) as a fixed independent factor, and species as a random independent factor nested within strategy. \*\*\* means significant with P < 0.001

	DF	F-ratio	P -value
<b>Roots</b>			
Strategy	1	13.7	0.015
Species (strategy)	4	6.1	< 0.001***
<b>Leaves</b>			
Strategy	1	0.1	0.82
Species (strategy)	3	7.4	< 0.001***

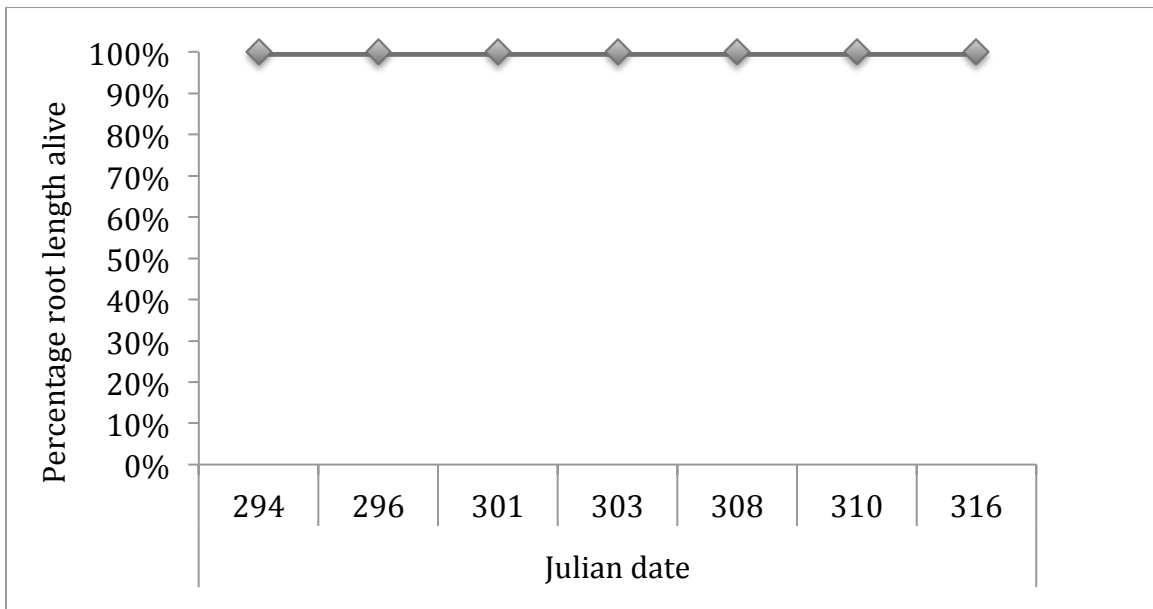
### 3.2. Root mortality

As seen in Figures 2 a and 2 b two species with perennial roots – *I. versicolor* and *S. microcarpus* – had 100% live roots in all dates of harvests (October 21 to November 12), based on vital staining using TTC. *C. oligosperma*, the third species with perennial roots had a slight decline of surviving roots, as indicated by decrease of stained roots down to 60% from 100% (Figure 2 c). This may also have been caused by penetration of the stain through the thick rhizodermis of this species. On the other hand, *S. americanum* and *R. alba*, both possessing annual root systems, had the highest mortality of roots comparing among all the species (Figures 2 d, e). *S. americanum* experienced a decline in surviving roots down to 30% within the harvesting period, whereas *R. alba* experienced a mortality of more the 90% mortality until the last harvest.

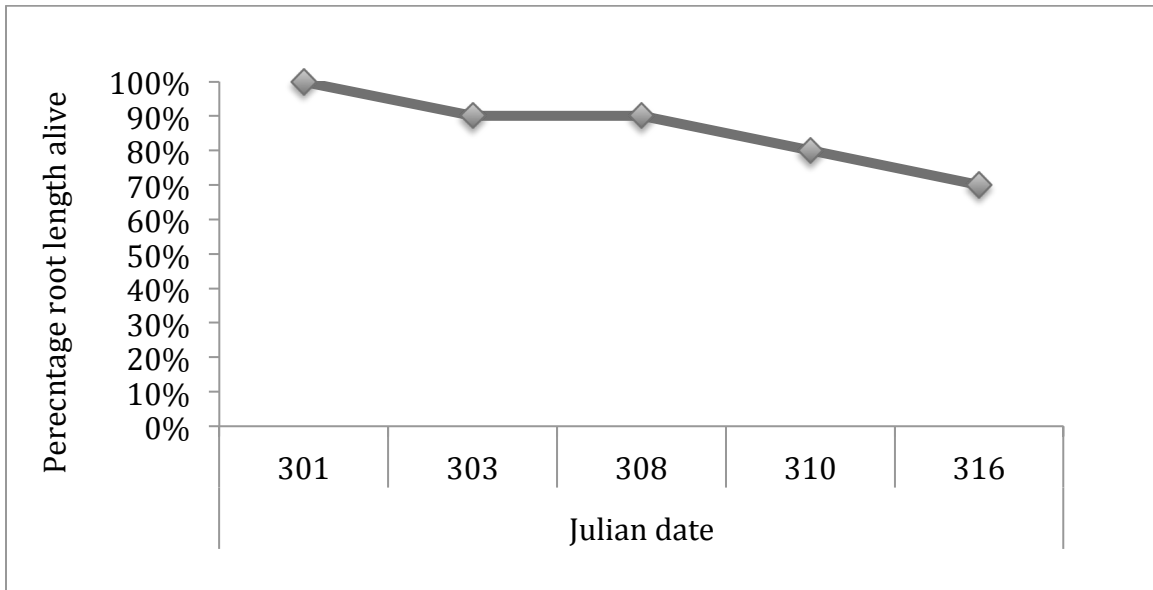
a) *Iris versicolor*



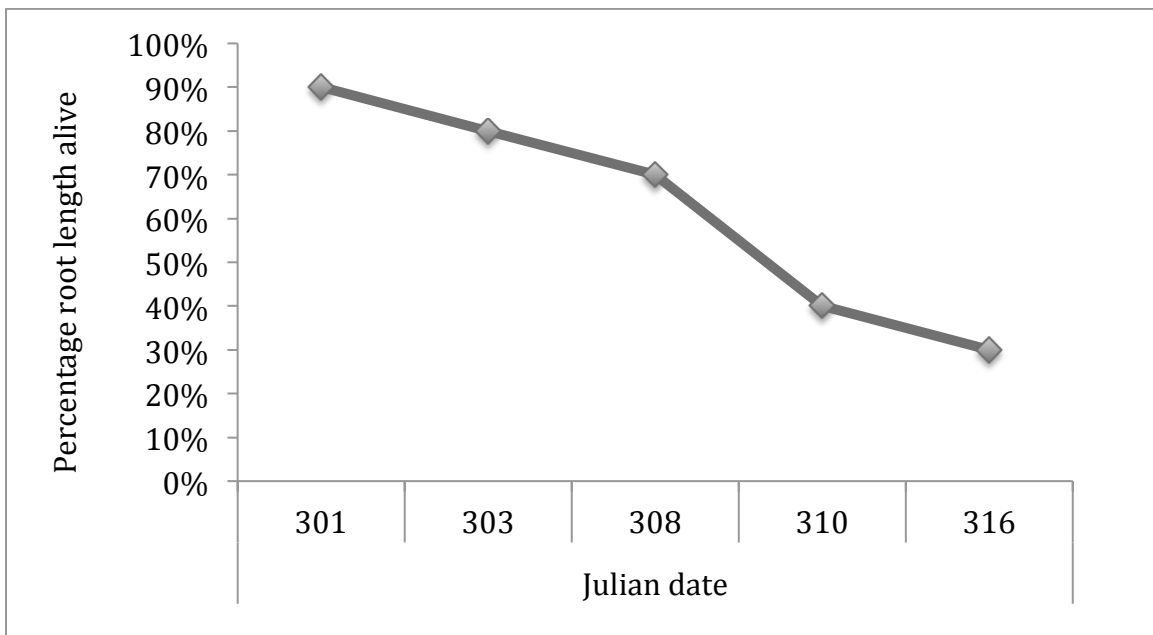
b) *Scirpus microcarpus*



c) *Carex oligosperma*

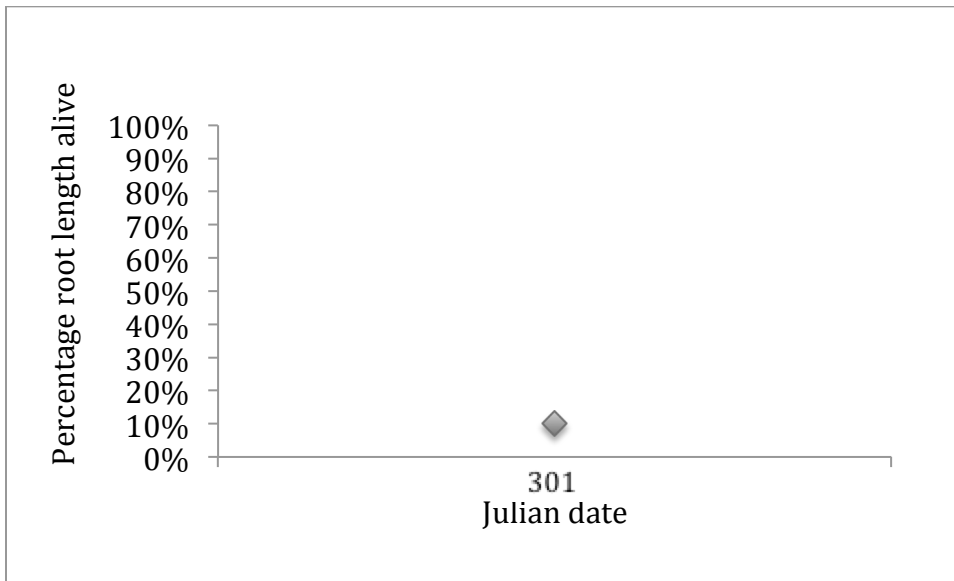


d) *Sparganium americanum*





e) *Rhynchospora alba*



**Figure 2:** Percentage alive root length in the roots of the studied species in the autumn. Root vitality was determined by staining with, 2, 3, 5-Triphenyl Tetrazolium Chloride (TTC). a-c: species with perennial roots, d-e: species with annual roots.

### 3.3. Protein content in roots and leaves

The protein content of the roots declined during fall in species with autumn-senescing root, such as *S. americanum*, *R. alba* and *S. latifolia* with a negative trend ( $p < 0.15$ ) over time for each of the species (Figure 3 a, b, c). However, protein content in roots for species with overwintering-roots system – *I. versicolor*, *S. microcarpus* and *C. oligosperma* did not show any significant trend (Figure 3 d, e, f). An ANOVA over all species showed a highly significant day  $\times$  strategy type interaction with p-value  $< 0.001$  (Table 4).

The protein content of the leaves of all species decreased during the fall, except in leaves of *C. oligosperma* (Figure 3). Testing these relationships with an ANOVA with strategy type and species nested within strategy types as independent factor, and day of harvest as a continuous independent variable, the effect of day was significant. Strategy type had no significant effect, but the day  $\times$  strategy type was weakly significant (Table 4).

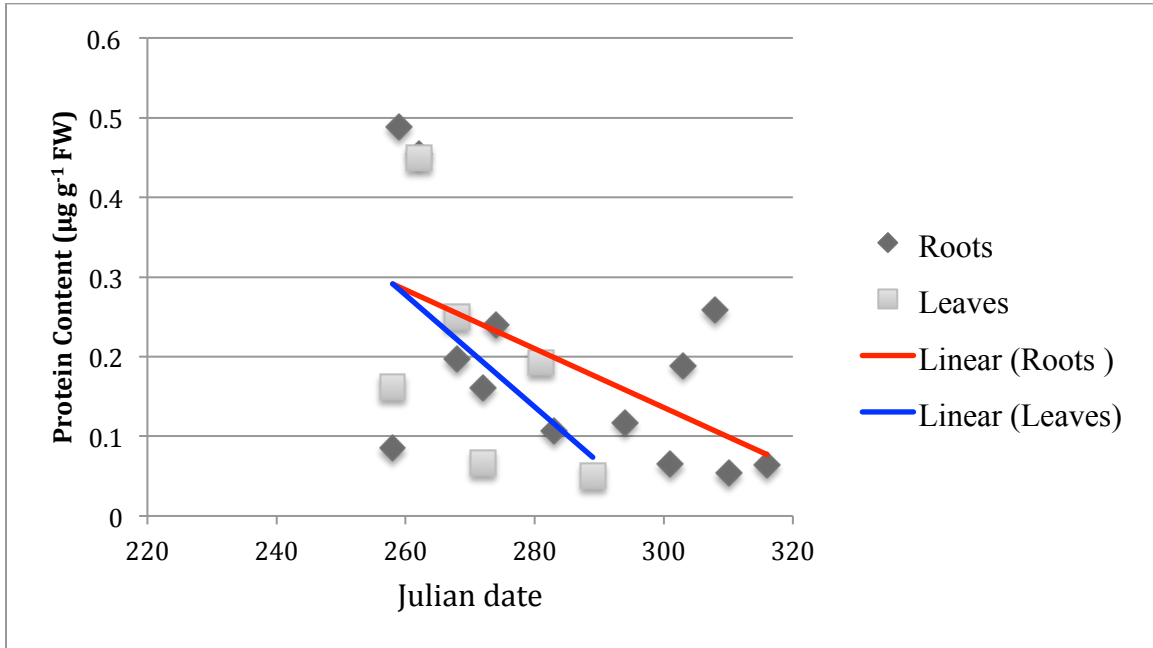
The protein content of the green overwintering bulbils of *R. alba* was measured on November. With  $2.7 \pm 0.4 \text{ mg g}^{-1}$  it was as high as in leaves before the senescence.

**Table 4.** Results of mixed model nested ANOVAs testing for leaf and root protein content per fresh mass. Protein content as the dependent variable, root strategy type (Strategy; annual roots, perennial roots) as fixed independent factor, and day of harvest (Day) as continuous independent variable. The model also contained species nested within strategy type as a random independent factorial variable. Number of observations: 75 for roots, 52 for leaves. Significance levels: \*\*\*  $P < 0.001$ , \*\*  $P < 0.01$ , \*  $P < 0.05$

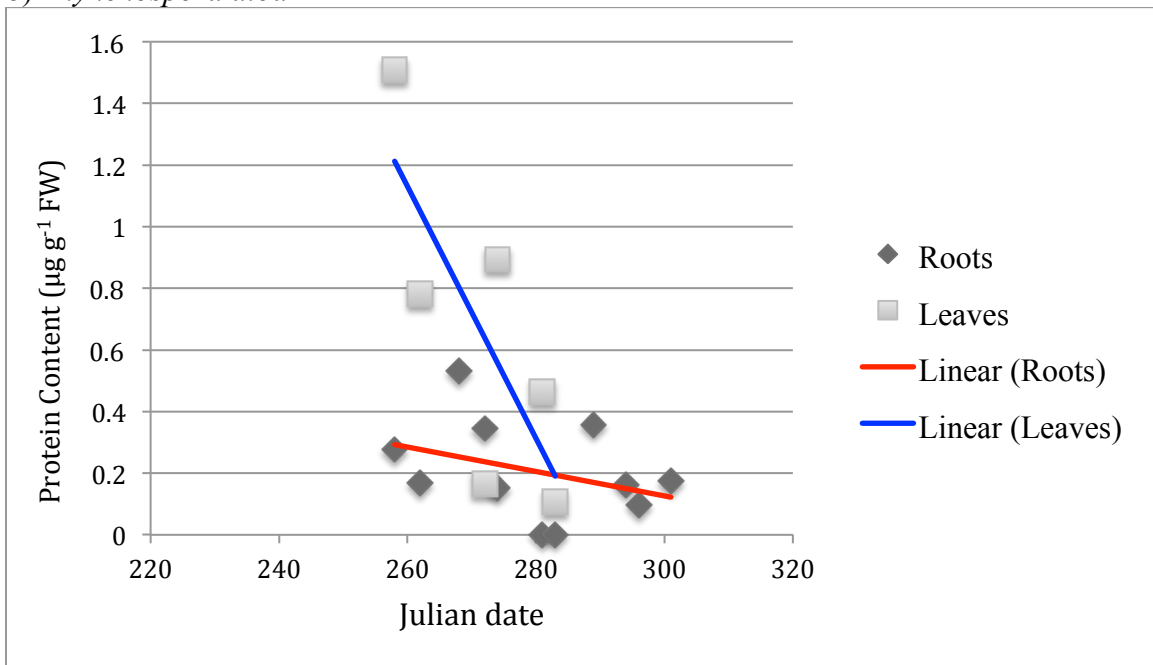
	F-ratio	P -value
Roots		
Strategy	2.157	0.215
Day	2.275	0.136
Day $\times$ Strategy	12.635	<0.001***
Leaves		
Strategy	0.659	0.462
Day	4.922	0.031*
Day $\times$ Strategy	6.015	0.018**

**Figure 3:** Leaf and root protein contents (in  $\text{mg g}^{-1}$  FW) of the six studied species plotted per fresh mass in course of autumn senescence. Regression lines indicated in blue (leaves) and red (roots). Solid lines  $p < 0.15$ , dotted lines  $p > 0.15$ . a-c: species with annual roots, d-f: species with perennial roots.

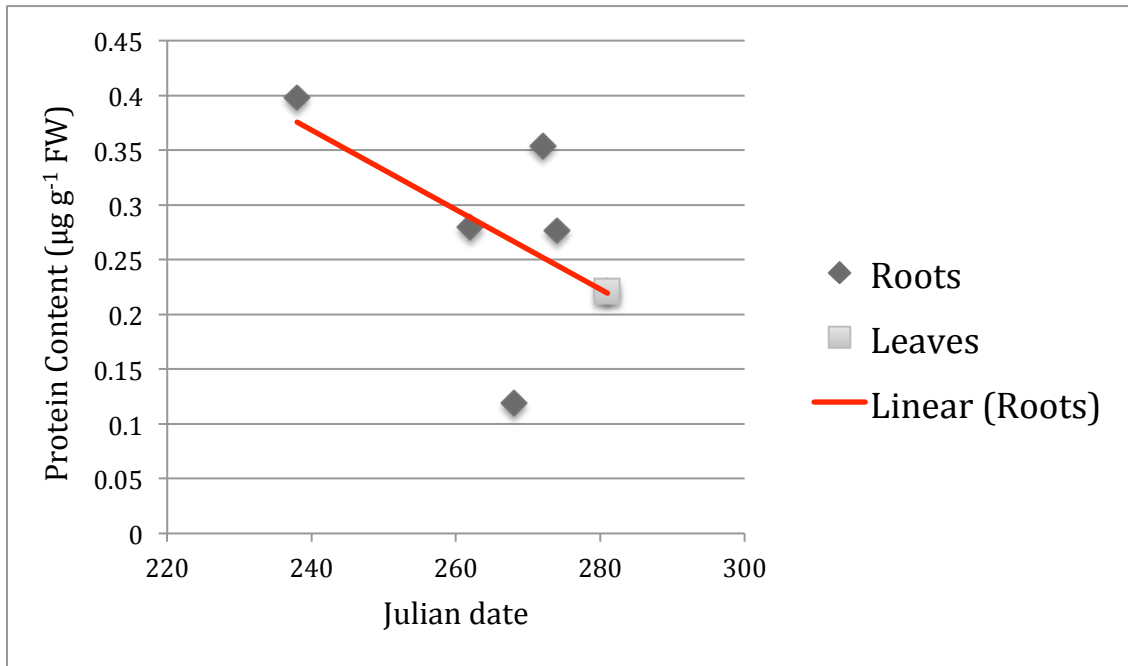
a) *Sparganium americanum*



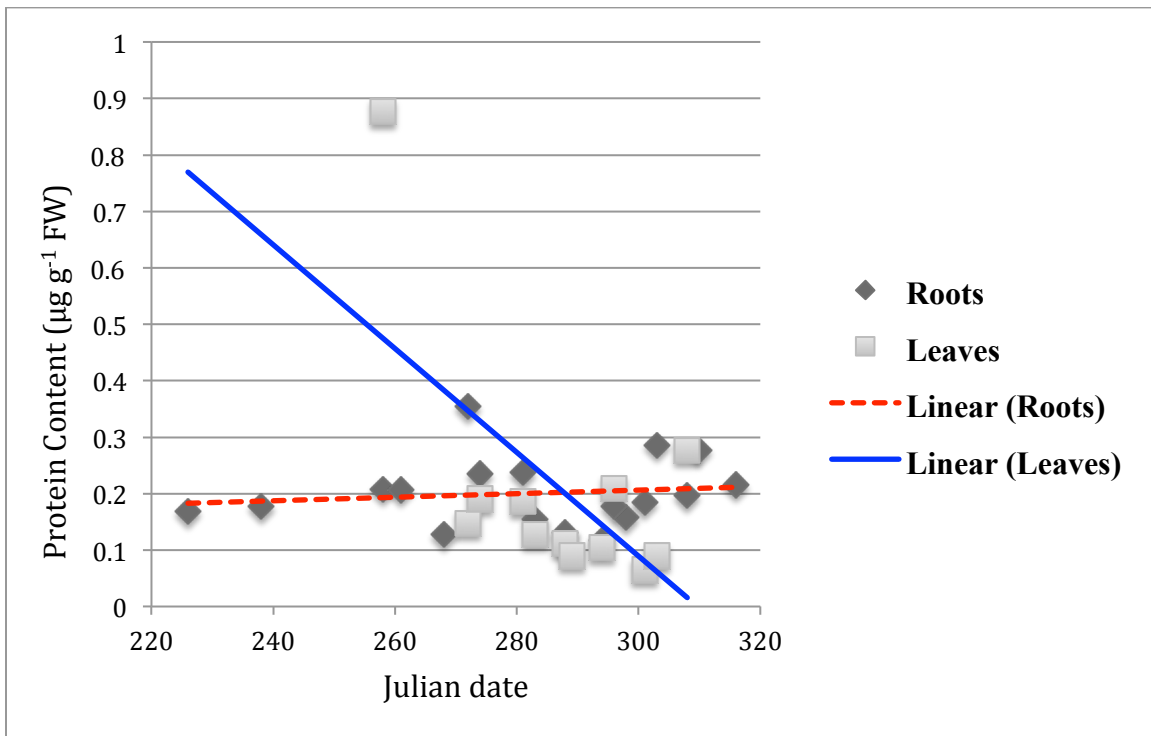
b) *Rhynchospora alba*



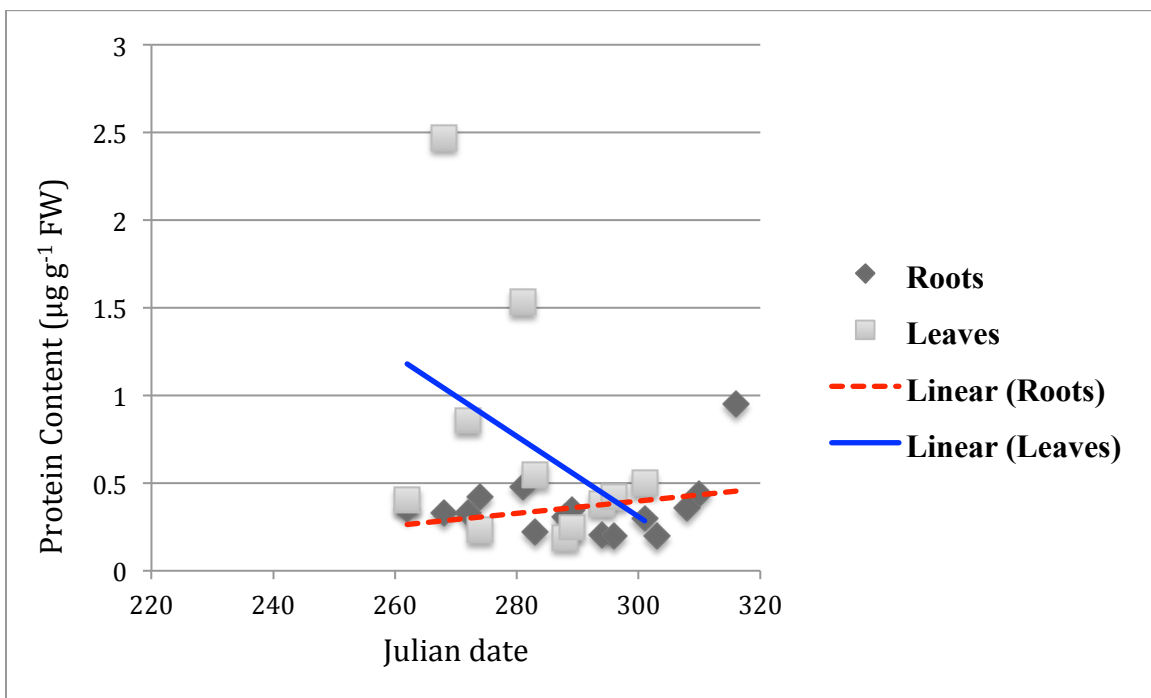
c) *Sagittaria latifolia*



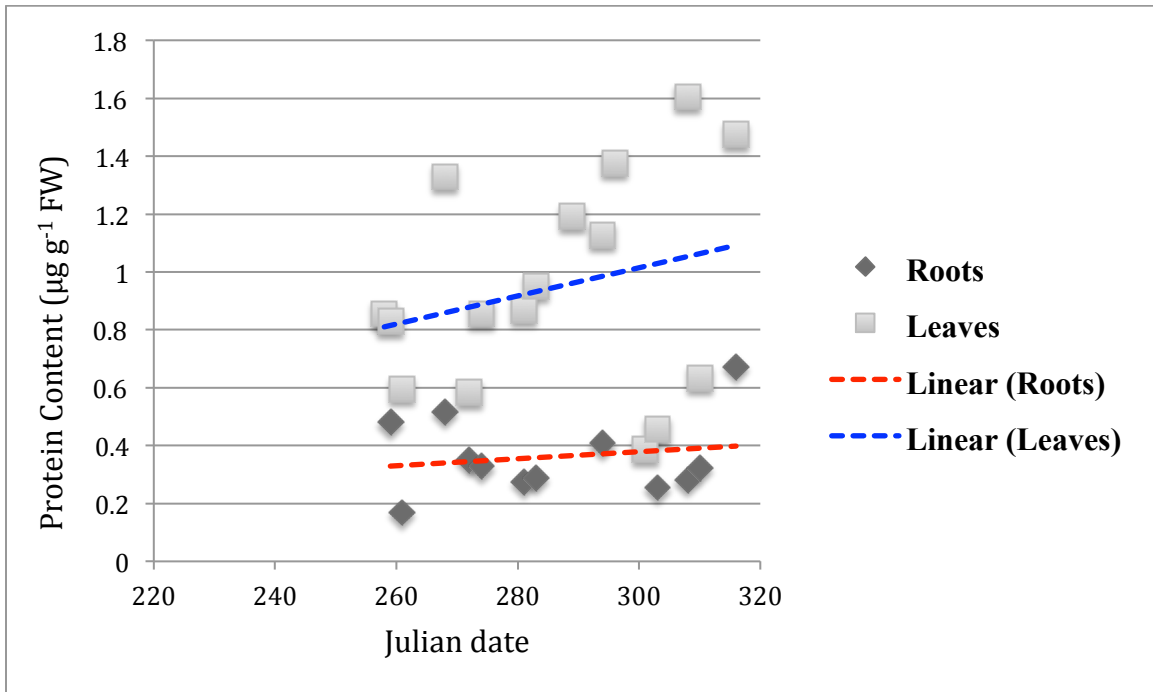
d) *Iris versicolor*



e) *Scirpus microcarpus*



f) *Carex oligosperma*



### 3.4. Leaf and root aminopeptidase activity

In linear regressions for each individual species, aminopeptidase activity per root fresh mass showed a negative trend ( $p < 0.150$ ) in species with annual roots (*R. alba*, *S. americanum* and *S. latifolia*) (Figures 4b, 5b, 6b), whereas for two of the species with perennial roots (*S. microcarpus* and *C. oligosperma*) there was no trend (Figures 8b, 9b). For the third species with perennial roots, *I. versicolor*, there was a slight positive trend (Figure 7b). Over all species, the effects of strategy type and day of harvest, were not significant, but their interaction was highly significant (Table 5) indicating a different response over time for the two strategies.

In individual regressions, aminopeptidase activity per leaf fresh mass showed only in *I. versicolor* and *R. alba* negative trends ( $p < 0.150$ ) over time (Figure 7b, 4b). In an ANOVA with all species neither the effects of strategy type, day of harvest nor their interaction were significant (Table 5).

Aminopeptidase within the roots corrected for their protein content showed a negative trend for two species with annual roots, *R. alba* and *S. latifolia* (Figure 4a, 6a). For leaves, *I. versicolor* showed a negative trend (Figure 7a). However, an ANOVA over all species did not show any significant effects, neither in roots nor in leaves (Table 6).

### 3.5. Leaf and root carboxipeptidase activity

The measured carboxipeptidase activities in leaves and roots were not distinguishable from controls, and hence not further analyzed.

**Table 5.** Results of mixed model nested ANOVAs testing leaf and root aminopeptidase activity per fresh mass. Aminopeptidase activity as the dependent variable, root strategy type (annual roots, perennial roots) as a fixed dependent factor, and species nested within strategy as a random independent variable. Number of observations: 65 for roots, 49 for leaves. \*\*\* Means significant with  $P < 0.001$

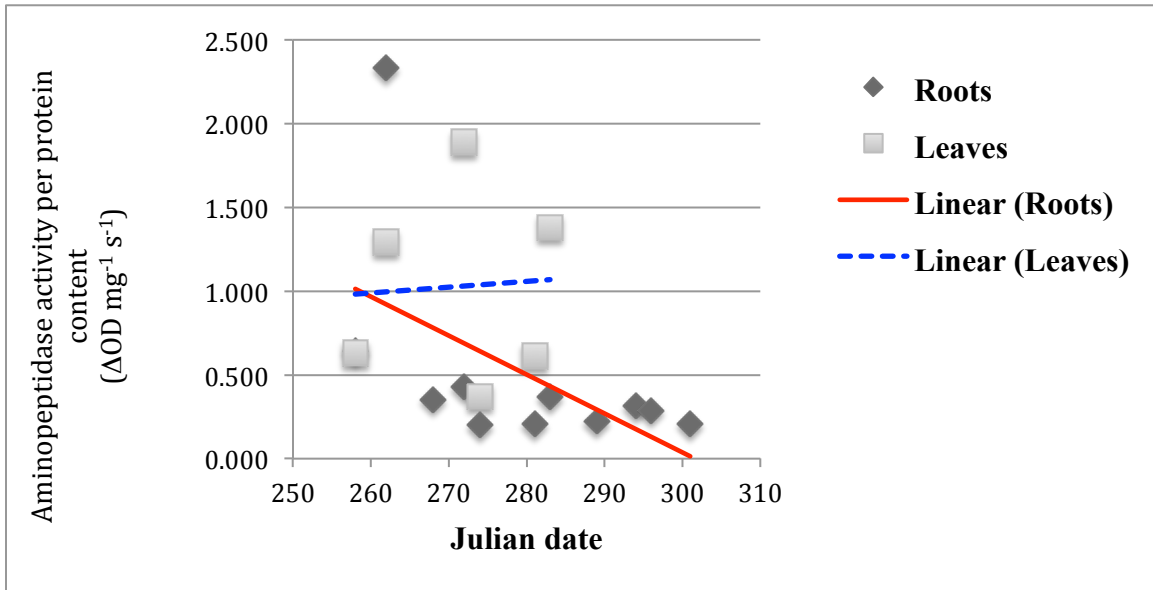
	<b>F-ratio</b>	<b>P -value</b>
<b>Roots</b>		
Strategy	0.449	0.539
Day	1.382	0.244
Day×Strategy	14.868	< 0.001***
<b>Leaves</b>		
Strategy	0.318	0.611
Day	0.489	0.487
Day×Strategy	0.400	0.530

**Table 6.** Results of nested ANOVAs (mixed model) testing leaf and root amino peptidase activity per protein content. Amino peptidase activity as the dependent factor, root strategy type (annual roots, perennial roots) as fixed independent factor, and species nested within strategy type as a random independent factor, and day of harvest as continuous independent variable. Number of observations: 65 for roots, 49 for leaves.

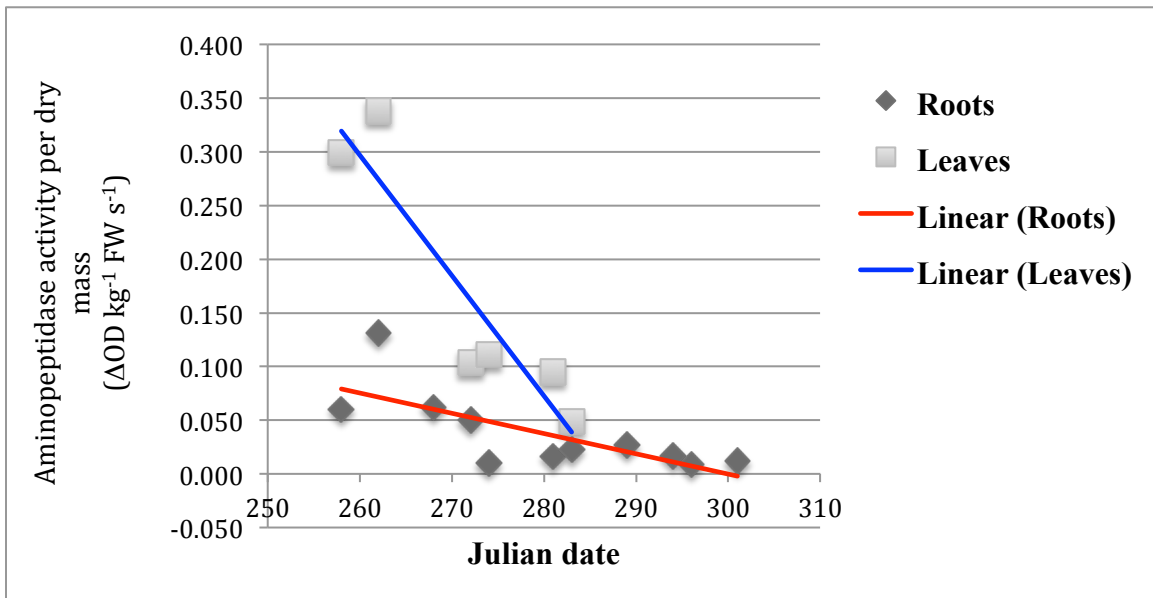
	<b>F-ratio</b>	<b>P -value</b>
<b>Roots</b>		
Strategy	0.035	0.860
Day	0.386	0.536
Day×Strategy	1.075	0.304
<b>Leaves</b>		
Strategy	0.054	0.830
Day	0.294	0.590
Day×Strategy	0.511	0.478



a)

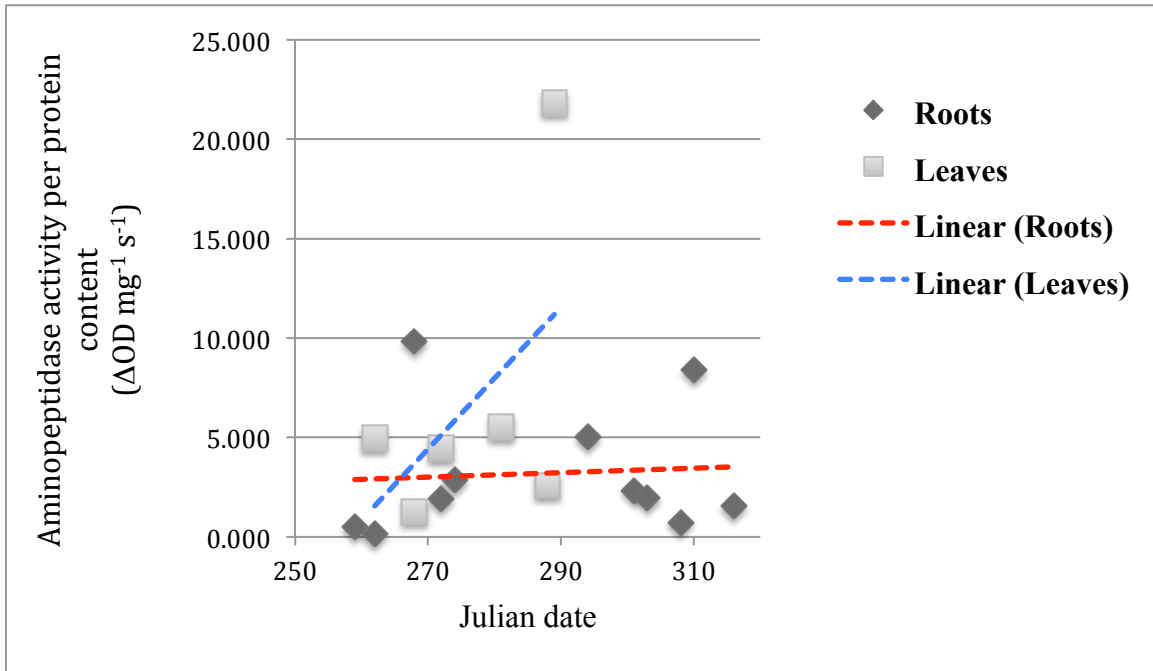


b)

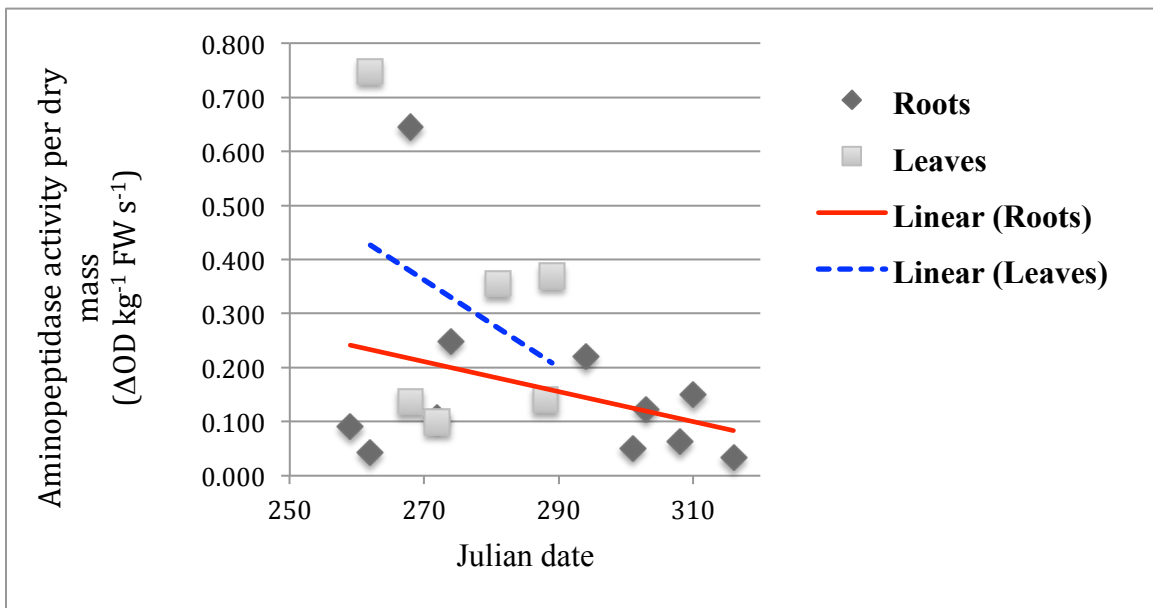


**Figure 4:** Leaf and root aminopeptidase activity for *Rhynchospora alba* plotted per fresh mass in course of autumn senescence. a) Aminopeptidase activity per protein content. b) Aminopeptidase activity per fresh mass. Regression lines indicated in blue (leaves) and red (roots). Solid lines  $p < 0.15$ , dotted lines  $p > 0.15$

a)

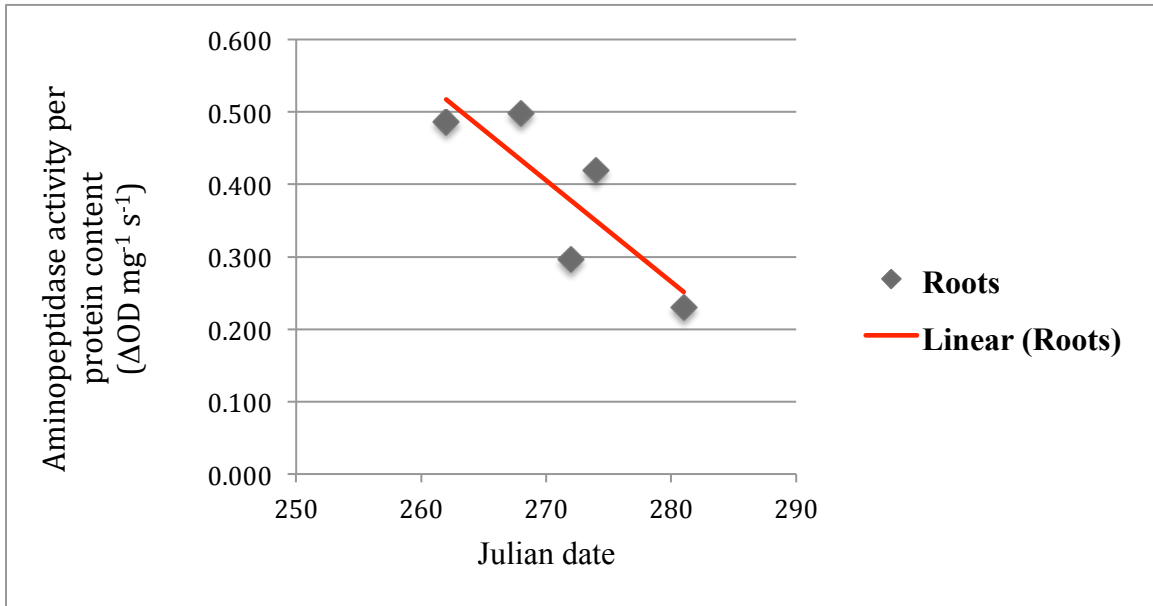


b)

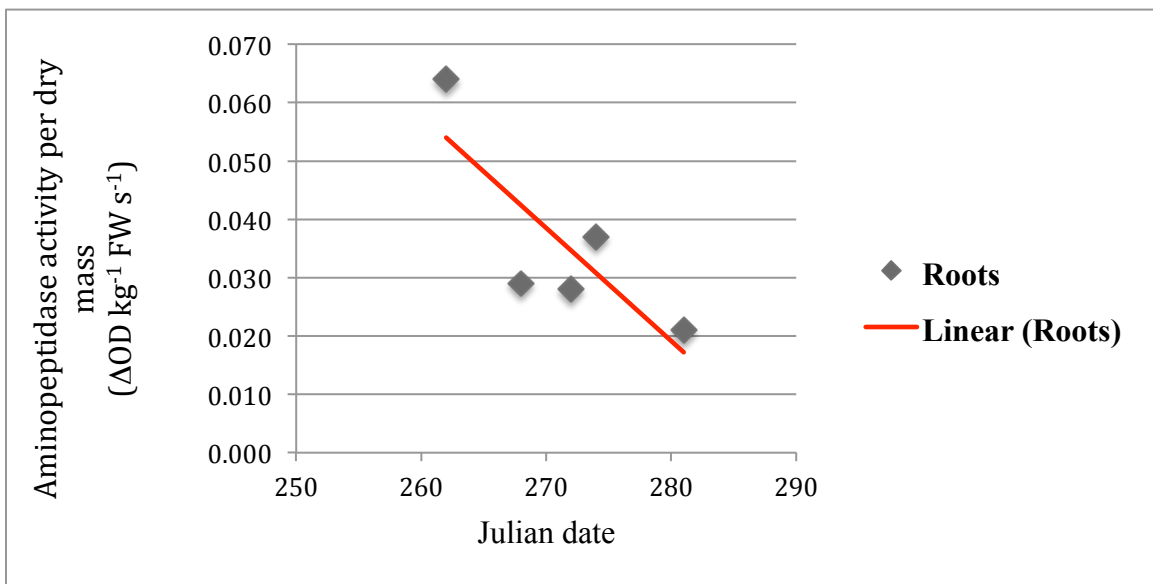


**Figure 5:** Leaf and root aminopeptidase activity for *Sparganium americanum* plotted per fresh mass in course of autumn senescence. a) Aminopeptidase activity per protein content. b) Aminopeptidase activity per fresh mass. Regression lines indicated in blue (leaves) and red (roots). Solid lines  $p < 0.15$ , dotted lines  $p > 0.15$

a)

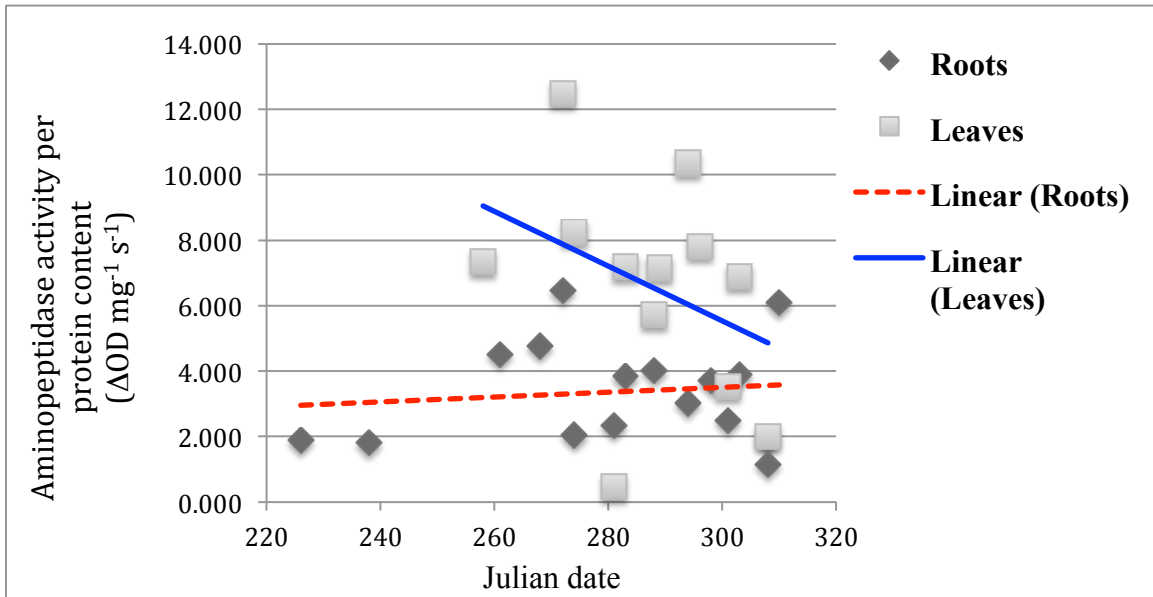


b)

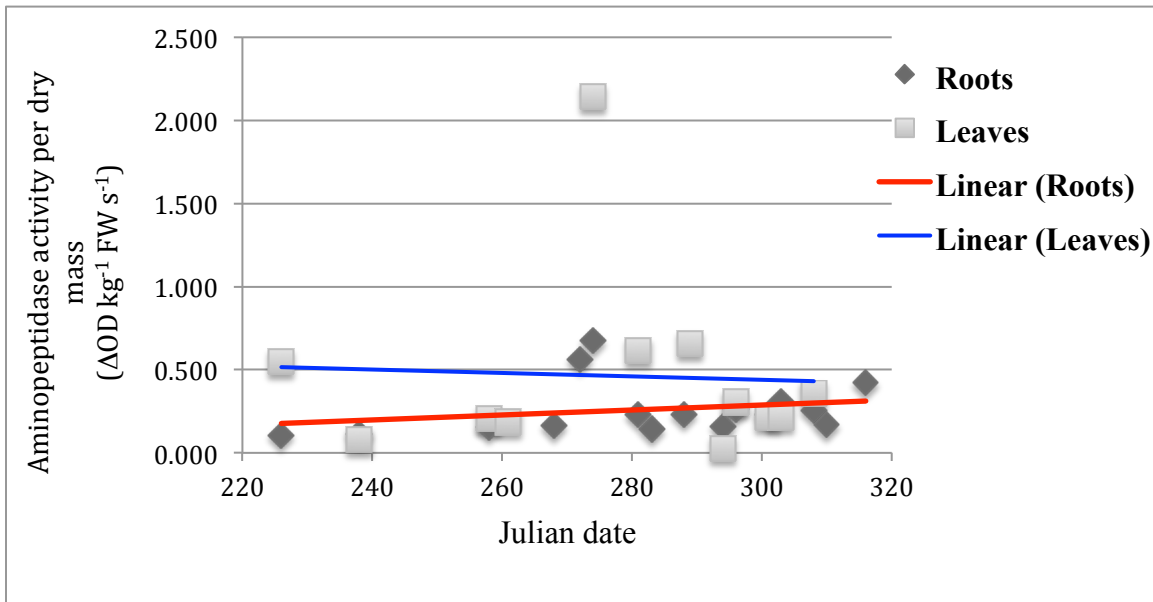


**Figure 6:** Root aminopeptidase activity for *Sagittaria latifolia* plotted per fresh mass in course of autumn senescence. a) Aminopeptidase activity per protein content. b) Aminopeptidase activity per fresh mass. Regression lines indicated in red (roots). Solid lines  $p < 0.15$ , dotted lines  $p > 0.15$ .

a)

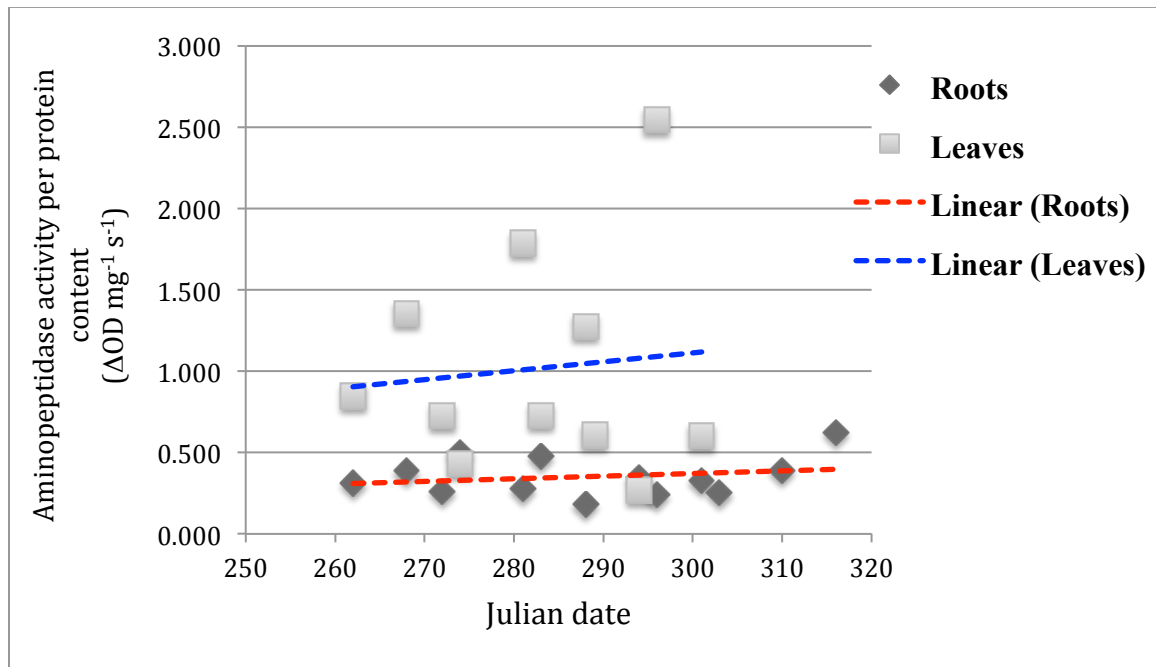


b)

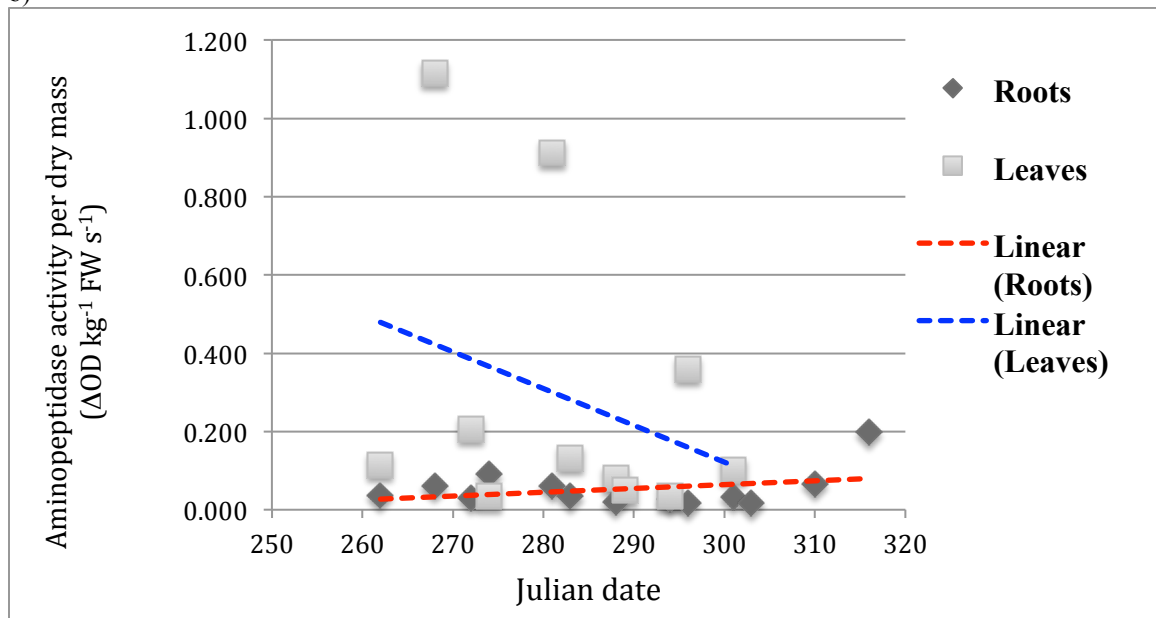


**Figure 7:** Leaf and root aminopeptidase activity for *Iris versicolor* in course of autumn senescence. a) Aminopeptidase activity per protein content. b) Aminopeptidase activity per fresh mass. Regression lines indicated in blue (leaves) and red (roots). Solid lines  $p < 0.15$ , dotted lines  $p > 0.15$ .

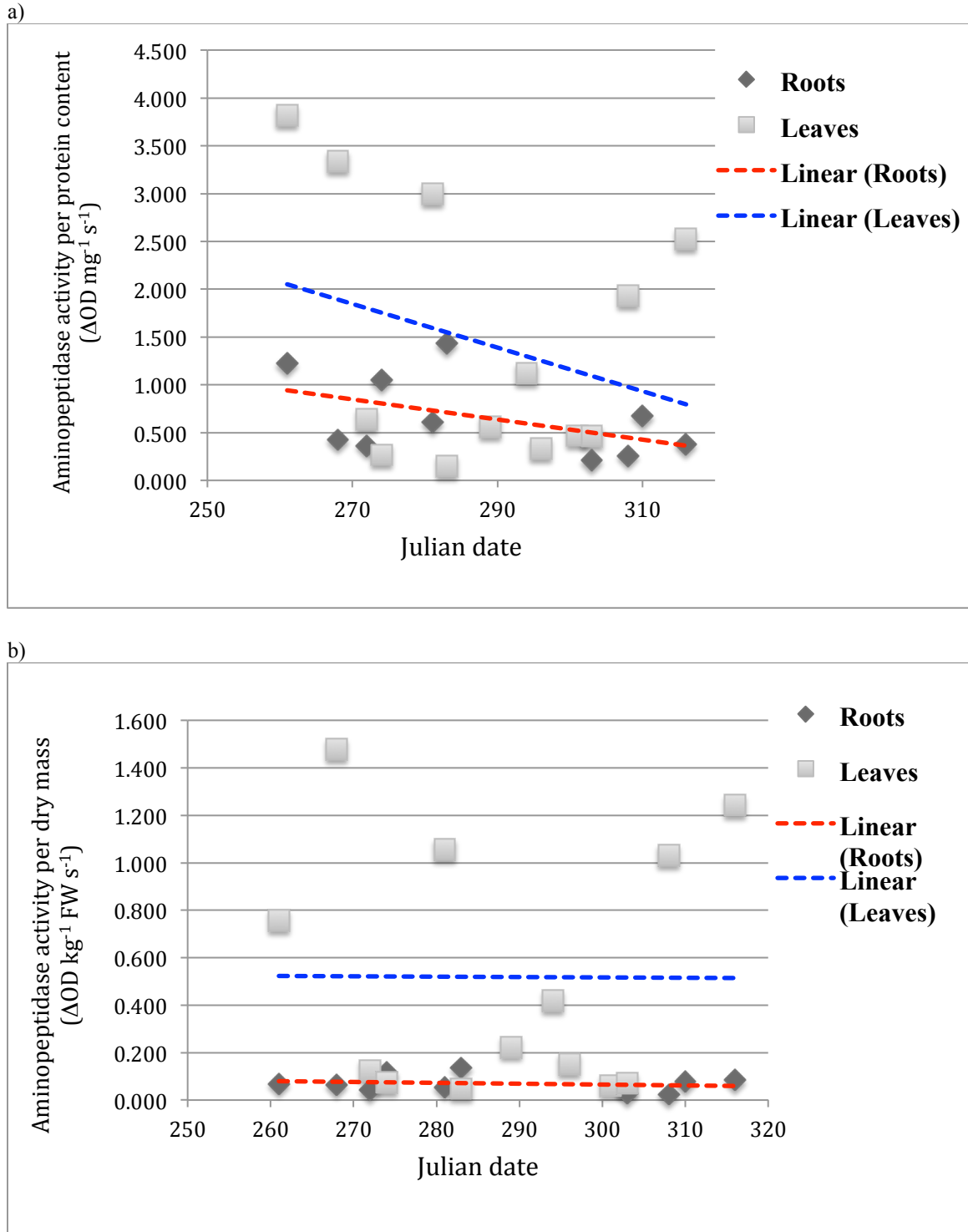
a)



b)



**Figure 8:** Leaf and root amino-peptidase activity for *Scirpus microcarpus* plotted per fresh mass in course of autumn senescence. a) Amino-peptidase activity per protein content. b) Amino-peptidase activity per fresh mass. Regression lines indicated in blue (leaves) and red (roots). Solid lines  $p < 0.15$ , dotted lines  $p > 0.15$ .



**Figure 9:** Leaf and root aminopeptidase activity for *Carex oligosperma* plotted per freshmass in course of autumn senescence. a) Aminopeptidase activity per protein content. b) Aminopeptidase activity per fresh mass. Regression lines indicated in blue (leaves) and red (roots). Solid lines  $p < 0.15$ , dotted lines  $p > 0.15$ .

## 4 Discussion

With respect to autumn senescence, the studied species behaved as expected. Leaves of all the studied species senesced during the harvesting period or before it, as in case of *S. latifolia*. Leaf senescence was observed as visual observation of chlorophyll degradation, and detected as a decline in leaf protein content in the leaves collected from all species, except in *C. oligosperma* (Fig. 3 F).

The root dry matter content (DMC) of species with perennial roots was significantly higher than that of species with annual roots, in accordance with previous findings (Gagnon 2014) and in agreement with the generally observed relationship between root life span and root dry matter content (Schlöpfer & Ryser 1996). This is expected given that the roots of perennial species probably have thickened cell walls (Wahl & Ryser 2000), and may also have a higher starch content as they are prepared for overwintering (Fonda & Bliss 1966). Roots of the annual species are present for the growing season only and will die, as indicated by the high mortality rates until November (70 and 90%) and the decline in protein content, and do not need to be sturdy to survive cold temperatures or to have any carbohydrate storage. Comparatively, only 0-30% mortality and no decline in protein content over the study period was detected in species with perennial roots. Hence, overall, roots in species expected to have annual roots showed indeed a stronger senescence than species that were expected to have overwintering roots.

Aminopeptidase activity per fresh mass also declined in the harvested leaves in this study. Protein degradation which is a senescence-linked metabolic activity that senescing plants employ to mobilize nitrogen and phosphorus from enzymatic and structural

proteins in senescing leaves in preparation for relocating them to other non-senescing parts of the plant. A similar trend was observed in the roots of species with annual roots. Aminopeptidase activity is reported to increase slowly during the reproductive stage and rapidly as leaves start senescence, while at the later stage of seed filling the activity decreases (Feller *et al.*, 1977). This finding suggests that the collection of the samples in the current study were taken in the late stages of senescence. If there was any peak in aminopeptidase activity at the beginning of the senescence, it might have been detectable if the sampling would have started earlier. Unfortunately most of our August samples were destroyed.

Overall, the decrease in aminopeptidase activity per fresh mass in both leaves and fine roots of species with annual roots matched the observed patterns of senescence. Specific aminopeptidase per protein content declined for most species, however, the trend was not significant.

#### **4.1. Review of nitrogen remobilization from leaves.**

Protein content has been reported to decrease in senescing leaves of plants (e.g.: Makino *et al.*, 1984, Hashimoto *et al.* 1989, Lutts *et al.*, 1996, Jiang *et al.*, 1999, Xu *et al.*, 2012). Protein degradation is one of the tasks during senescence that cells undergo as they naturally age or reach the end of the growth season, or when induced by an external factor, such as nutrient deficiency, shade, extreme temperatures, drought, and pathogenic infection, ending in programmed cell death (Gan and Amasino, 1997). Treating with ethylene can also induce senescence (Grbić and Bleecker, 1995), indicating that ethylene is involved in the signaling. Protein in leaves is largely associated with photosynthetic



machinery, but leaves can also be regarded as nitrogen storage organs during their vegetative growth phase and until they reach full expansion, at which point degradation of Rubisco becomes a source of amino acids required at sink organs and continues to do so through senescence (Thomas, 2013). In animals senescence is usually associated with wear and tear, but in plants it is a tightly regulated active process as a part of the plant's development as an adaptation to their environment, responsible for minimizing resource losses by remobilizing before the organ is being discarded (Thomas, 2003; Masclaux-Daubresse & Krupinska, 2014).

Protein content in senescing leaves changes in two ways: the decrease of protein content as the proteins are degraded to amino acids and remobilized elsewhere (Hashimoto *et al.*, 1989), and the *de novo* synthesis of new proteolytic and other enzymatic proteins required for the degradation of the leaf protein for facilitating other biochemical processes during the programmed cell death (Feller *et al.*, 1977 & 2007; Roberts *et al.*, 2012).

Nitrogen, a limiting element and growth determinant in most ecosystems (Aerts and Chapin, 2000), is a macronutrient that the plant invested energy in absorbing from the soil through the roots against its natural concentration gradient across the cell membranes. Nitrogen is mainly present in the leaf cells in the form of proteins especially those associated with carbon fixation in photosynthesis, and chlorophyll. However, only protein nitrogen can be recycled as the chlorophyll nitrogen is not remobilized and it is lost when leaves are shed despite its partial degradation (Liu *et al.*, 2008, Yang *et al.*, 2004). Therefore, protein degradation during senescence is most needed by the plant as a

method of mobilizing the nitrogen (Côté *et al.*, 2002; Avila-Ospina *et al.*, 2014). During senescence the catabolic activities in the senescing leaves work on mobilizing the nitrogen stored in those leaves proteins (regarded as the source) through breaking the proteins down to free amino acids via the proteolytic enzymes (Bhalerao *et al.*, 2003, Guo *et al.*, 2004). The freed amino acids are translocated to varying storage parts, developing organs, or seeds of the plant (regarded as the sink), depending on the considered species and the causes of senescence, to be used in synthesizing new storage, structural, and enzymatic proteins (Thomas, 2013; Roberts *et al.*, 2012).

Kamachi *et al.* (1991) reported a fourfold increase in the synthesis of *de novo* cytosolic glutamine synthetase in rice plants during senescence accompanied by an increase in glutamine content, suggesting that the freed glutamate (the major form of free amino acids in rice leaves) from the protein degradation process in senescing leaves are prepared for mobilization by transforming it to glutamine. Glutamine and asparagine are often regarded as the main molecules for nitrogen transports in plant due to their higher N content, but other studies have shown that freed amino acids could be transported without any modifications (Liu *et al.*, 2008).

Protease and peptidase activities have been reported as a major contributor in the nitrogen remobilization in senescing leaves since chlorophyll nitrogen does not make it to a remobilized form (Yang *et al.*, 2004, Parrot *et al.*, 2005, Liu *et al.*, 2008, Roberts *et al.*, 2012). Aminopeptidase surge in activity was found to be associated with senescence in corn leaves when estimated in alkaline conditions (pH 7), while carboxypeptidase activity was found to increase when estimated at acidic pH (3-6) (Feller *et al.*, 1977).

By studying the endopeptidase, amino- and carboxypeptidase activities and their transcript levels a strong correlation between the induction of senescence and the up-regulation/increased activity of some of the studied enzymes have been reported (Yang *et al.*, 2004, Parrot *et al.*, 2005). Similar results were obtained for different types of proteases in the same studies. Roberts *et al.* (2012) concluded that a wide range of proteases are involved in senescence in leaves based on the evidence that their expression in senescing leaves is up regulated. In the vacuole, exo and endo proteases, peptidases, endopeptidases, and aminopeptidases carry out the protein degradation after being transported into the vacuole from other organelles, specially the chloroplasts (Liu *et al.*, 2008).

## **4.2 Review of nitrogen remobilization from roots**

Senescence in roots has been less studied, and not much is known about the protein content change during senescence in the roots. Studying remobilization of nitrogen and other nutrients from roots is complicated by the fact that roots can also be a sink that receives recycled macronutrients, especially when root systems survive the winter (Rossato *et al.*, 2001). Nambiar (1987) reported no significant variation in nitrogen content of pine roots with seasonal changes as opposed to their fluctuating carbohydrate content over the same period, neither was there a difference in nitrogen content of live and dead roots. Given that pine is an evergreen tree, relying on Nambiar's data to exclude any senescence related changes in nitrogen content of roots or their remobilization could be misleading. Simpson *et al.* (1983) reported that in wheat, which is an annual species with terminal senescence of the whole plant, the roots contributed 16% of the total protein in grains. Freschet *et al.* (2010) found a decreased N and P content in dead roots

of subarctic plants, but the results remain inconclusive as the exact time of death was not known, and the decrease might have happened as leaching after the death. Interestingly, Fisher *et al.* (2002) reported that there is no evidence of programmed death in roots of common bean plants, which undergo a terminal senescence at the end of the year, and added that soil microorganisms are more likely the cause of death of the roots after the shoots have senesced. However, the study was done on bean plants grown under phosphorous deficiency conditions, which may have changed the patterns of senescence.

### **4.3 Comparison of findings of this thesis with literature**

In the current study the protein content of the harvested leaves of the studied plants behaved according to what has been reported in the literature. The decrease in protein content of leaves during senescence has been reported as a second phase of senescence during the seed filling process in annual plants, following an increase in the protein content at the beginning of the senescence (Feller *et al.*, 1977). Leaves of all studied species senesce for the winter, and correspondingly protein contents in all leaves decreases. An exception was *Carex oligosperma* with no decrease in leaf protein content, but this species is known to have late senescing leaves (Ryser & Kamminga 2009), and they were indeed fully green until the end of this study. When this is compared with the findings of the current studies it is perceived as that the decrease in the senescing leaves of 4 of the studied species coincided with remobilization of nutrients to overwintering organs before leaf death. For *S. latifolia* and *R. alba* the remobilization might also have been associated with seed filling. There was slight increasing trend in the protein content of *C. oligosperma* which may have coincided with the initiation of senescence.

In species with annual roots, root protein content decreased parallel to leaf protein content, indicating the senescence of all vegetative parts except that of the perennating organ, rhizome in case of *S. americanum*, tubers in case of *S. latifolia* and bulbils at the stem base in case of *R. alba*. In species with overwintering roots root protein content did not decrease. The overwintering bulbils of *R. alba* remained green and had a high protein content even in November.

This decrease in fine root protein content with simultaneous aminopeptidase activity suggests a behaviour in roots which can be considered as active senescence, protein degradation in order to remobilize the nutrients to be stored over winter. This is the first report of a decrease in protein content during autumn senescence in roots. Izumi *et al.* (2015) showed that plastids are recycled during energy-limitation induced autophagy in rice roots, which suggests that there could be protein degradation in the roots associated with senescence.

In contrast to expectations, leaf aminopeptidase activity was not significantly changing over time. As protease genes are being activated when leaves start to senesce (Buchanan-Wollaston 1997), one might expect protease activity to go up. This has been found for example by Anderson & Rowan (1965) in *Nicotiana tabacum* detached leaves. However, this situation may not be representative for natural seasonal senescence, as N cannot be transported out of the leaves. In general, association between protein degradation and protease activity is not straightforward, and studies have found positive, negative and missing correlations (Huffaker 1990). The results may depend on type of senescence and treatment, as during dark-induced senescence endoproteolytic activity

increased in detached barley leaves, while attached leaves did not showed a slight decrease (Huffaker 1990). Aminopeptidase activity can also be associated with other than programmed senescence e.g. with cadmium-induced stress in root tissues (Boulila-Zoghلامي *et al.*, 2011).

Roots of species with annual roots showed a decrease in aminopeptidase activity in course of the autumn, in contrast to roots of species with long-lived roots. An expected increase of aminopeptidase activity at the beginning of the autumn was not observed, but this may have been a result that the first harvest in mid September was already too late, senescence may have started earlier. It has also been reported that as the roots of maize age aminopeptidase activity declines during seed filling stage after an initial increase in activity during the development of tassels and ears (Feller *et al.*, 1977). Furthermore, autophagy of whole root plastids in rice was detected and photographed (Izumi *et al.*, 2015), which is a similar behaviour to that of chloroplasts in senescing leaves (Carrión *et al.*, 2014; Wada and Ishida, 2009). These data, together with the results of the current study suggest a programmed senescence in the roots.

Avila-Ospina *et al.* (2015) stated that vacuole endopeptidase and the autophagy pathway are most probably the responsible mechanism for chloroplast protein degradation during senescence, and that both carboxypeptidase and endopeptidase activities in acidic pH are established markers of leaf senescence and nitrogen remobilization. Also a cysteine endopeptidase was reported to be involved in programmed cell death, as in pathogen-induced senescence, and is only found in plants (Helm *et al.*, 2008; Höwing *et al.*, 2014). Moreover, the activity of endopeptidase was

reported to increase as senescence develops, parallel to a decrease in exopeptidase (both carboxy- and aminopeptidase) activity in maize leaves (Feller *et al.*, 1976). Another suggesting evidence in favour of senescence in fine roots comes from the decreased nitrogen content of the senescing fine roots of perennial tree species 12-28% as corrected for mass loss (Kunkle *et al.*, 2009). The correction for mass loss calculation method was used in order to get more representative data since the total mass loss in the senescence season was a factor in showing a false increase in nitrogen content. However, the study of Kunkle *et al.* (2009) did not include any confirmation of the fate of the lost nitrogen.

In the current study the data of specific aminopeptidase activity. i.e., the activity corrected for the total protein content were not consistent with the absolute aminopeptidase activity per fresh mass. This could be explained as that the proteolytic activity of the aminopeptidase and other decreases along with the total protein content, i.e., with structural and other enzymatic proteins.

## **5. Conclusion**

The results confirm the existence of two types of root turnover strategies among Northern Ontario wetland monocots: species with root which senesce in autumn, and species with roots which survive the winter. These strategy types were associated with different seasonal patterns of root protein content, species with senescing roots showing declining protein contents. This strongly supports that the dying roots indeed have active senescence processes going on, leading to remobilization of nitrogen. Amino-peptidase activity was found during the protein content decrease, but the expected increase in specific activity was not found. However, comparison with literature shows that a correlation between declining protein content and protease activity is not always easy to find.



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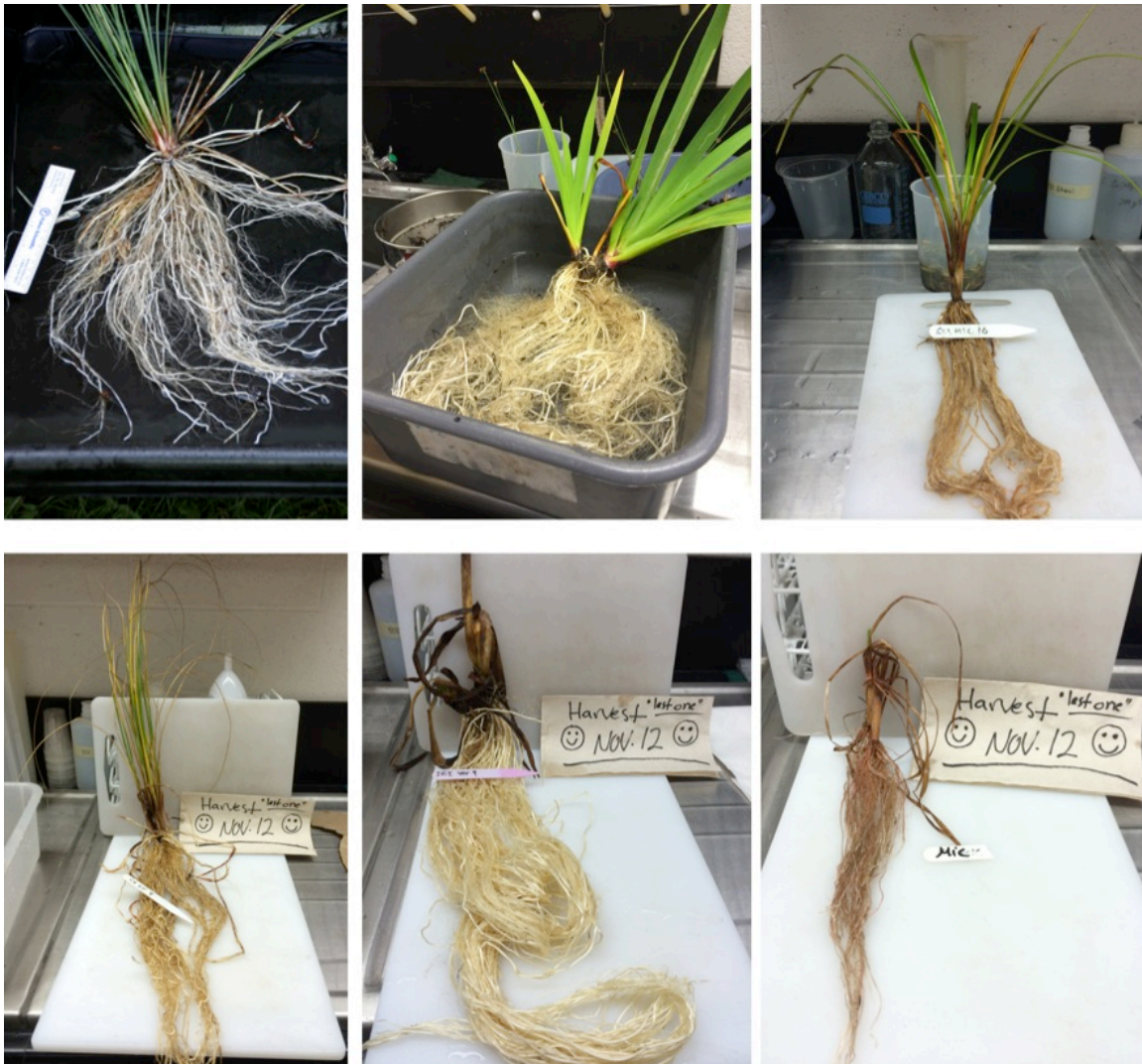
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**Appendix 1.** Pictures for the garden at the beginning of the experiment in May (a), at beginning of harvesting in September (b) and at setting up the experiment in May (c).



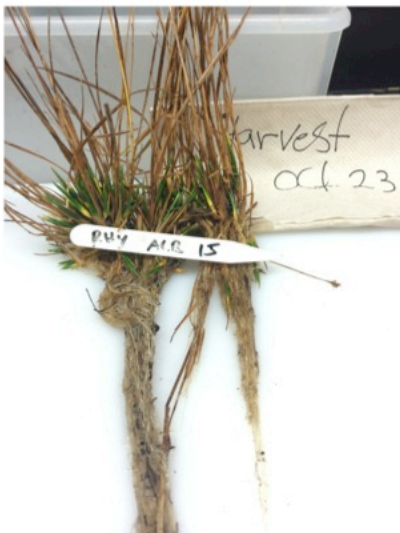
**Appendix 2.** Pictures for roots and leaves of all species at early and late harvests.

- Three species with perennial roots





-Three species of annual roots



**Appendix 3:** Pictures of roots after staining with TTC test in November at the last harvest to investigate root mortality.

